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An epidemiological inquiry

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MARSHAL MUTINDA MWEU | PHD THESIS 2013

Streptococcus agalactiae infection in the population of Danish dairy cattle herds: An epidemiological inquiry

Streptococcus agalactiae infection in the population of Danish dairy cattle herds: An epidemiological inquiry

Marshal Mutinda Mweu

PhD thesis 2013

This thesis has been submitted to the Graduate School of the Faculty of Health and Medical Sciences, University of Copenhagen

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“Of making many books there is no end, and much study wearies the body”

~ *Eccl.* 12:12

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Lastly, upon stumbling on the timeless phrase, "If you can't win, don't lose", I, as scores of others beforehand, had hurriedly dismissed it as an oddity. Indeed, at first glance it sounded so, but pondering further I figured that, "if you won't lose" then at some point, "you will win". Unwittingly, Thomas Edison's life epitomised this philosophy – who when asked how he felt after having made 1,000 unsuccessful attempts at inventing the light bulb was unhesitant to point out that he never perceived them as failures but mere steps towards his decisive goal. Having thankfully borrowed a leaf from his viewpoint early on in my PhD, I realised that the key to successfully overcoming hurdles along the tortuous research path was carefully concealed in a case of constant self-evaluation and affirmation – prerequisites for keeping an unrelenting spirit. To this end, I can attest to the fact that persistence eventually rewards handsomely, if given a chance.

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SUMMARY

Streptococcus agalactiae (*S. agalactiae*) is a contagious pathogen that presents a noteworthy economic threat to the dairy industry owing to its deleterious effect on bovine udder health that is marked by significant productivity and milk quality problems. A surveillance and control programme for *S. agalactiae* was initiated in 1966 with the ultimate goal of eliminating infections with the pathogen from the population of Danish dairy herds. Considerable strides attributable to the control programme were made towards reducing and maintaining the frequency of infections at reasonably low levels up till 2000, when subsequently a re-emergence of the pathogen was noted. This has rekindled an interest in the epidemiology of the pathogen especially in view of the growing body of evidence implicating humans in new herd infections. Consequently, the current PhD project was instigated with the purpose of examining the epidemiology of the pathogen in Danish dairy herds in order to obtain information on performance of the available diagnostic tests, frequency and transmission characteristics of the pathogen, contact patterns of the cattle herd population and spatiotemporal patterns of infection. Knowledge of these aspects will be essential in facilitating *S. agalactiae* control decisions in the country.

The thesis is organised into 6 chapters:

In the initial part of chapter 1, a brief introduction of the aspects to be covered, the purpose of the PhD project, the objectives and the pertinent research questions that the thesis seeks to address are presented. In the second part, a review of existing literature on the epidemiologic triad for *S. agalactiae* infections, the impact of the infections, herd-level detection and control methods available as well as the infection situation in Denmark and other countries is given.

In chapter 2, the sensitivity (Se) and specificity (Sp) of both the conventional bacteriological culture and the PathoProof Mastitis PCR tests of bulk tank milk (BTM) samples are estimated while assessing the herd-level covariate effects of herd size, production type and milking system on the accuracies of the tests using a latent class analysis in a Bayesian framework. It was shown that the Se of the PCR assay was maximised at the cycle threshold (Ct) value of 40, at which the Se of PCR was found to be higher (95.2%; 95% posterior credibility interval [PCI] [88.2% – 99.8%]) than that of culture (68.0%; 95% PCI [55.1% – 90.0%]), although the Sp of culture was higher (99.7%; 95% PCI [99.3% – 100.0%]) compared to that of PCR (98.8%; 95% PCI [97.2% – 99.9%]). Further, the PCR test was shown to outperform culture in detection of herds with low levels of infection but culture demonstrated superiority in the diagnosis of herds with high levels of infection. The performances of the tests were found to be unaffected by the herd-level covariates. To optimise the identification of infected herds for control, it was recommended that surveys of BTM for *S. agalactiae* be based on the PCR assay with samples recording Ct values below 40 being scored positive. Nevertheless, it was advised that herds with Ct values close to the proposed cut-off be confirmed by culture in order to minimise the false positive fraction.

The study in chapter 3a sets out to estimate the annual herd-level incidence rates and apparent prevalences of *S. agalactiae* in Danish dairy herds over a 10-year period from 2000 to 2009 as well as demographic

parameters (herd-level entry and exit rates), the transmission parameter, β and the recovery rates for the infection over the same period. Increasing trends in both the incidence and prevalence of the infection were observed, with a surge in incidence being conspicuously notable in 2005. Per 100 herd-years the value of β was 54.1 (95% CI 46.0-63.7); entry rate 0.3 (95% CI 0.2 – 0.4); infection-related exit rate 7.1 (95% CI 5.6 – 8.9); non-infection related exit rate 9.2 (95% CI 7.4 – 11.5) and recovery rate 40.0 (95% CI 36.8 – 43.5). These parameters corresponded to an R_o value of 1.1 affirming the rise in incidence. To lower the frequency of infections, it was suggested that existing controls against *S. agalactiae* be strengthened.

In chapter 3b, a Bayesian framework is employed to estimate the annual herd-level true incidence risks and prevalences of *S. agalactiae* infection in the population of Danish dairy herds during the period spanning 1966 to 2011. It was shown that during the culture-based phase of the surveillance programme i.e. 1966 to 2008, the apparent prevalences (Ap) consistently underestimated the true prevalences (Tp), while contrastingly the apparent incidence risks (A_i) overestimated the true incidence risks (T_i) of the infection. During the PCR surveillance phase however, it was demonstrated that the Aps and the A_is overestimated the Tps and T_is of the infection respectively. Notably, fluctuations in the Tp and T_i trends closely mimicked changes in legislation pertaining to *S. agalactiae* control. Trends in the Tp and T_i declined following the incorporation of PCR into the surveillance of BTM in 2009. Bacteriological culture demonstrated suitability for use in diagnosing new herd infections, but was rather insufficient for detecting existing infections. By contrast, the PCR assay showed adequacy for identifying both new and existing herd infections thus offering promise for facilitation of on-going control efforts aimed at eliminating the infection from the population.

In chapter 4, the network of Danish cattle movements is characterised over the period from 2000 to 2009 with a view to establishing the connectedness of the network, influential holdings and the structural vulnerability of the network. As a whole, 50,494 premises were involved in 4,204,895 individual movements. The entire study period was partitioned into 120 monthly windows, each forming individual networks. Based on these monthly networks, it was observed that the overall network was predominantly scale free, although marked by small-world properties in March and April 2001 as well as in 24 other months during the period extending from October 2006 to December 2009. Moreover, the network was sparsely connected implying that an epidemic was likely to spread minimally locally, but rapidly through it. Markets were found to be the most influential holdings in the network enjoying a disproportionate share of the contacts. The vulnerability of the network to removal of markets suggested that targeting highly connected holdings during epidemics ought to be the focus of control efforts.

The study in chapter 5 seeks to describe the spatiotemporal patterns of infection with *S. agalactiae* in the population of dairy herds during the period from 2000 to 2009 and further, to estimate the annual herd-level baseline and movement-related risks of the infection over the same period. The results indicated that the risk of becoming infected with *S. agalactiae* varied spatiotemporally, with the risk being more homogeneous and higher after 2005. The annual baseline risks gave significant yet distinctive patterns before and after 2005; the risk being greater in the latter period. Contrastingly, the annual movement-related risks were non-significant over the 10-year period. Notably, there was neither evidence for spatial clustering of cases relative to the

population of herds at risk nor spatial dependency between herds. Nonetheless, the results signalled the need to step up within-herd biosecurity in order to minimise the risk of introducing the pathogen into naïve herds.

In chapter 6 a general discussion is presented not only clarifying issues arising from previous study chapters but also giving a new dimension to the results. Subsequently, conclusions and future perspectives are given.

It can be deduced from this thesis that the switch from bacteriological to PCR examination of the BTM affords better promise for detecting both new and existing herd infections and could thus effectively support control efforts aimed at eliminating *S. agalactiae* infections from the Danish dairy herd population. Furthermore, with the increasing role of human strains in the overall burden of infection, there is a pressing call for authorities to intensify awareness campaigns with the goal of increasing the uptake of: (1) within-herd biosecurity measures pertaining to hand sanitation, (2) the five-point plan for mastitis control, in particular with respect to post-milking teat disinfection and dry cow therapy and (3) strict observance of environmental hygiene to reduce the potential risk of environment-associated *S. agalactiae* infections.

SAMMENDRAG

Streptococcus agalactiae (*S. agalactiae*) er et smitsomt patogen, der er en anseelig økonomiske byrde for mejeriindustrien som følge af skadesvirkningerne i forbindelse med kvægets yversundhed, idet der sker en nedsat produktivitet og mælkekvalitetsproblemer i yveret. I 1966 blev der etableret et *S. agalactiae* overvågnings- og saneringsprogram, der havde som endeligt mål at udrydde infektioner med dette patogen fra den danske malkekvægspopulation. En række saneringstiltag blev effektueret og resulterede i en reduceret infektionshyppighed, som blev fastholdt på et lavt niveau indtil år 2000, hvorefter der skete en tilbagevenden af patogenet. Herved vendte interessen for patogenets epidemiologi tilbage, specielt som følge voksende evidens, der antydede at mennesker kunne spille en rolle i de nye besætningsinfektioner. En konsekvens heraf var etableringen af nærværende ph.d. projekt, der havde til formål at undersøge epidemiologien af patogenet i den danske malkekvægspopulation, med henblik på at erhverve information om de tilgængelige diagnostiske tests ydeevne, hyppighed af og spredningskarakteristika for patogenet, kontaktmønstre i kvægpopulationen, og de spatiotemporale infektionsmønstre. Kendskab til disse aspekter vil være essentielle for at fremme beslutninger vedrørende sanering for *S. agalactiae* i landet.

Afhandlingen er opdelt i 6 kapitler:

I indledningen af kapitel 1 gives en kort introduktion til de i afhandlingen beskrevne aspekter, ligesom ph.d. projektets formål, mål og relevante hypoteser præsenteres. Efterfølgende præsenteres en oversigt over den eksisterende litteratur indenfor den epidemiologiske triade for *S. agalactiae* infektioner, effekten af infektionerne, infektionspåvisning på besætningsniveau og tilgængelige saneringsmetoder, ligesom forekomsten i Danmark og andre lande beskrives.

I kapitel 2 estimers sensitiviteten (Se) og specificiteten (Sp) af både den konventionelle bakteriologiske dyrkningsmetode og PathoProof Mastitis PCR testen, begge anvendt på tankmælk, med samtidig evaluering af effekten af besætningsstørrelse, produktionstype og malkesystem på testsensitivitet og -specificitet i en Bayesiansk latentklasse model. Det blev vist, at PCR Se blev maksimeret ved en cyclus tærskelværdi (Ct) på 40, ved hvilken PCR Se blev estimeret til at være højere (95.2%; 95% posterior credibility interval (PCI) [88.2% – 99.8%]) end for dyrkning (68.0%; 95% PCI [55.1% – 90.0%]), om end Sp for dyrkning var højere (99.7%; 95% PCI [99.3% – 100.0%]) end den var for PCR (98.8%; 95% PCI [97.2% – 99.9%]). Endvidere blev PCR testen fundet at være bedre til at påvise lavere infektionsniveauer, mens dyrkningstesten var bedst til at diagnosticere besætninger med højere niveau. Testenes diagnostiske evne blev ikke påvirket af kovariaterne. For at optimere identifikationen af de smittede besætninger, blev det anbefalet, at overvågning af *S. agalactiae* i tankmælksprøver baseres på PCR testen, idet Ct-værdier under 40 klassificeres som positive. Samtidig anbefales det dog, at besætninger med Ct-værdier tæt på den foreslående cut-off bekræftes med dyrkning for at minimere den falskpositive fraktion.

Studiet i kapitel 3a var en estimering af de årlige besætningsincidensrater og tilsyneladende prævalenser af *S. agalactiae* i de danske malkekvægsbesætninger over en 10-års periode fra 2000 til 2009, sammen med estimater for de demografiske parametre (opstarts- og ophørsrater på besætningsniveau),

smittespredningsparametren β og helbredelsesraterne over den samme periode. Der blev set en tendens til stigende infektionsincidens og –prævalens, med en påfaldende kraftig stigning i 2005. Per 100 besætningsår var β 54.1 (95% konfidensinterval (KI) 46.0-63.7); opstartsraten 0.3 (95% CI 0.2 – 0.4); infektionsrelateret ophørsrate var 7.1 (95% KI 5.6 – 8.9); ikke-infektionsrelateret udgangsrate var 9.2 (95% KI 7.4 – 11.5) og helbredelsesrate var 40.0 (95% CI 36.8 – 43.5). Disse parametre svarede til en R_o på 1.1, hvilket bekræftede incidensstigningen. Det blev foreslået, at de eksisterende kontrolforanstaltninger skulle strammes for at reducere infektionshyppigheden.

I kapitel 3b blev en Bayesiansk tilgang anvendt for at estimere de årlige sande besætningsincidensrisikoer og -prævalenser af *S. agalactiae* infektion i den danske malkekvægspopulation i perioden fra 1966 til 2011. Det blev vist, at i den periode, hvor bakteriologisk dyrkning blev anvendt til overvågning, dvs. fra 1966 til 2008, blev den sande prævalens (TP) konsekvent underestimeret ved den tilsyneladende prævalens (Ap). I modsætning hertil blev de sande infektionsincidensrisikoer (T_i) overestimeret af de tilsyneladende infektionsincidensrisikoer (A_i). Det er bemærkelsesværdigt, at fluktuationstendenserne i TP og T_i følger forandringerne i den gældende lovgivning vedrørende *S. agalactiae*. Der var tendens til fald i TP og T_i følgende introduktionen af PCR til overvågning i 2009. Bakteriologisk dyrkning har vist sig at være passende til at påvise nye infektioner, men utilstrækkelig til at påvise højprævalente infektioner. Til sammenligning er PCR metoden både tilstrækkelig til at påvise nye og eksisterende infektioner, hvilket er lovende i de saneringstiltag, som er rettet mod at udrydde infektionen fra populationen.

I kapitel 4 karakteriseres netværket af kvægflytninger i Danmark for perioden omfattende årene 2000 til 2009 med henblik på at beskrive sammenhæng, de mest indflydelsesrige enheder og den strukturelle svaghed af netværket. Samlet set var 50.494 enheder involveret i 4.204.895 individuelle flytninger. Hele studieperioden blev opdelt i 120 månedsvinduer, der hvert dannede et individuelt netværk. Baseret på disse månedlige netværk kunne det observeres at det samlede netværk stort set var skaleringsfrit, selvom der i perioden marts til april 2001 og over 24 måneder i perioden fra oktober 2006 til december 2009 var markante antydninger af smågrupperinger. Desuden var netværket sparsomt forbundet, hvilket antyder at en epidemi mest sandsynligt vil spredes minimalt lokalt, men hurtigt vil kunne spredes igennem netværket. Markeder blev fundet at være de mest indflydelsesrige enheder i netværket, da de står for en uforholdsmæssig stor andel af kontakterne. Netværkets svaghed for fjernelsen af markederne antyder, at disse bør vælges som hovedindsatsområder ved en epidemi.

Studiet i kapitel 5 søger at beskrive de spatiotemporale mønstre ved *S. agalactiae* infektioner i malkekvægspopulationen i 2000-2009 og desuden at estimere de årlige baseline- og flytningsrelaterede infektionsrisikoer på besætningsniveau i den samme periode. Resultaterne indikerede at risikoen for at blive smittet med *S. agalactiae* varierede spatiotemporalt, idet risikoen var mere homogen og højere efter 2005. De årlige baselinerisikoer viste signifikante og samtidig distinkte mønstre før og efter 2005, med højst risiko i den sidstnævnte periode. I modsætning hertil var de årlige flytningsrelaterede risikoer non-signifikante over den 10-årige periode. Det er bemærkelsesværdigt, at der ikke var evidens for hverken spatiel klyngedannelse af cases i forhold til den modtagelige population eller spatiel afhængighed mellem besætningerne. Ikke desto

mindre signalerer resultaterne et behov for opstramning af de interne smittebeskyttelsestiltag for at reducere introduktion af patogenet i fuldt modtagelige besætninger.

I kapitel 6 præsenteres en generel diskussion, der ikke alene afklarer indholdet fra de tidligere kapitler, men også giver en ny dimension af resultaterne. Efterfølgende gives konklusioner og fremtidsperspektiver.

Det kan udledes fra denne afhandling, at skiftet fra bakteriologisk dyrkning til PCR undersøgelser af tankmælksprøver giver lovning om bedre påvisning af både nye og eksisterende besætningsinfektioner, hvormed PCR analyserne effektivt kan støtte saneringstiltagene, der retter sig mod at udrydde *S. agalactiae* infektioner fra den danske malkekvægspopulation. Derudover er der med den stigende rolle af de humane stammer i den samlede infektionsbyrde et presserende behov for, at myndighederne intensiverer kampagner for at fremme opmærksomheden med henblik på at fremme brugen af: (1) interne smittebeskyttelsestiltag omkring håndhygiejne, (2) fempunktsplanen for mastitissanering, specielt hvad angår pattedesinfektion efter malkning og goldkobehandling, og (3) mere stringent overvågning af miljøhygiejnen for at reducere potentiel miljøassocierede *S. agalactiae* infektioner.

CHAPTER 1:

GENERAL INTRODUCTION AND LITERATURE REVIEW

1.1 GENERAL INTRODUCTION

1.1.1 Overall introduction

Streptococcus agalactiae (*S. agalactiae*) is a contagious pathogen that detrimentally impacts bovine udder health resulting in productivity and milk quality losses (Keefe, 1997). In Denmark, a surveillance and control scheme for *S. agalactiae* has been in existence since 1966 (Anon., 1966-1982). The control programme had, for a significant period of time, dramatically reduced and maintained the infection frequency at fairly low levels in the dairy herd population (Anon., 1980-1992). However, since 2000, a resurgence of the pathogen has been noticed that warrants an investigation into its epidemiological characteristics in the population, its effective detection and aspects of the population that could facilitate the control of its spread.

Since the inception of the Danish *S. agalactiae* surveillance programme, bacteriological culture of bulk tank milk (BTM) samples has been the standard diagnostic tool. Plagued by a low sensitivity, culture of the BTM necessitates longitudinal testing to enhance the detection of infected herds (Godkin and Leslie, 1993). With the introduction of the novel, real-time PathoProof Mastitis PCR assay into the surveillance scheme, there is promise for improved identification and control of infection owing to its capability in detecting growth-inhibited bacteria (Koskinen et al., 2009). Nevertheless, there is need to evaluate the assay's field performance and hence its potential for routine use in *S. agalactiae* screening. Further, availability of covariate information such as herd size, milking system and type of production may facilitate an assessment of their effect on the performance of the test.

The effectiveness of the *S. agalactiae* control programme can be assessed based on an understanding of the incidence, prevalence and transmissibility of the pathogen. The magnitude of the basic reproductive ratio together with trends in the pathogen frequency may allude to possible breakdowns in *S. agalactiae* control, probable evolution of the pathogen toward increased antimicrobial resistance and transmission or spill over of human *S. agalactiae* into the bovine population (Zadoks et al., 2011). However, in order to reveal true trends in frequency, an adjustment for test misclassification in both incidence and prevalence estimates is necessary.

Understanding the contact structure of the Danish cattle herd population may offer meaningful insights into the determination of the most effective way of applying control strategies that could minimise the spread of *S. agalactiae*. It has been observed that contact networks endowed with holdings that have substantial in and out animal movements are exceedingly prone to spread and persistence of infections. These highly connected holdings are often at risk of contracting and transmitting infections and are thus considered to exhibit super-spreader behaviour (Keeling and Eames, 2005). Therefore, the success of control efforts in such networks is seen to rely on targeted (as opposed to random) application of control measures on these holdings (Rautureau et al., 2011).

An assessment of the relative roles of within-and between-herd risk factors in spurring new *S. agalactiae* infections may support the effective prioritisation of resources by disease control personnel. Moreover, a spatiotemporal analysis could be used to demonstrate the geographical extent and the temporal variation in risk patterns that could provide the basis for ruling out practices that facilitate local transmission of the

infection while also highlighting the impact of changes in the adoption of on-farm biosecurity measures on the risk of infection with the pathogen.

1.1.2 Purpose of the PhD project

To investigate the epidemiology of *S. agalactiae* in the population of Danish dairy herds with a view to obtaining key information on the accuracy of available diagnostic tests, frequency and transmission characteristics of the pathogen, contact structure of the cattle herd population and spatiotemporal patterns of infection with the pathogen. An understanding of these aspects will be central to informing decisions aimed at optimising *S. agalactiae* control in the national dairy herd population.

1.1.3 Objectives and research questions

1. To estimate the herd sensitivity (Se) and specificity (Sp) of bacteriological culture and PathoProof Mastitis PCR using latent class models in a Bayesian analysis, while evaluating the effect of herd-level covariates on the Se and Sp of both tests. The corresponding questions are:

- How does the performance of the novel PCR assay compare with that of the conventional bacteriological culture?
- Does the PCR assay lend itself to routine use in *S. agalactiae* screening programmes?
- Are the accuracies of the tests affected by the size of individual herds, type of production and milking system?

2a. (i) To estimate the annual herd-level incidence rates and apparent prevalences of *S. agalactiae* in the population of Danish dairy herds over a 10-year period from 2000 – 2009 inclusive.
(ii) To estimate the herd-level entry and exit rates (demographic parameters), the transmission parameter, β, and recovery rate for *S. agalactiae* infection. The corresponding question is:

- How frequent and transmissible is *S. agalactiae* in the population and do these traits signal a possible re-emergence of the pathogen?

2b. To estimate the annual herd-level true incidence risks and prevalences of *S. agalactiae* in the population of Danish dairy herds during the period from 1966 – 2011 inclusive. The corresponding question is:

- What is the significance of the diagnostic misclassification in the control of *S. agalactiae* in Danish dairy herds and has there been a change in *S. agalactiae* frequency since the incorporation of PCR testing of BTM samples?

3. To characterise the network of Danish cattle movements over a 10-year period from 2000 – 2009 with a view to understanding: (1) cohesiveness of the network, (2) influential holdings and (3) structural vulnerability of the network. The corresponding questions are:

- What is the overall contact pattern of the Danish cattle herd population and its bearing on simulation modelling of the effectiveness of control strategies against *S. agalactiae*?
- What is the implication of the presence of highly connected holdings in the network of Danish cattle herds on the risk of infection with *S. agalactiae*?

4. (i) To describe the spatiotemporal patterns of infection with *S. agalactiae* in the population of Danish dairy herds from 2000 – 2009.

(ii) To estimate the annual herd-level baseline and movement-related incidence risks of *S. agalactiae* infection over the 10-year period. The corresponding questions are:

- How is the pattern of infection in the population distributed both in space and time?
- Is there evidence for the local spread of *S. agalactiae* in the population of dairy herds?
- What are the relative roles of within-and between-herd factors in driving *S. agalactiae* infections in the population?

1.2 LITERATURE REVIEW

1.2.1 The epidemiologic triad for *S. agalactiae* infections

The epidemiologic triad is a classical model framework of disease causation. An intricate interplay of its three components (vertices) - the agent, host and environment – is often responsible for precipitating disease in the host (Fig.1). Hereunder, the framework is employed to facilitate the description of the mechanism of *S. agalactiae* infections.

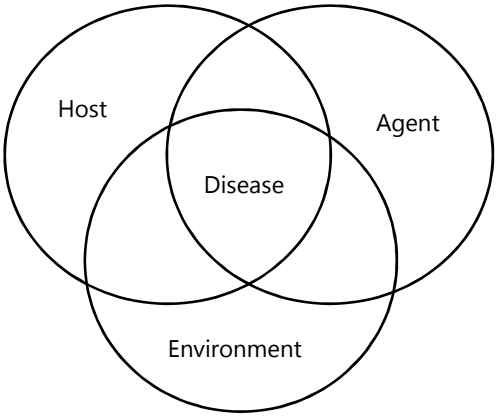

Fig. 1. The epidemiologic triad illustrating the agent-host-environment interaction in disease causation.

1.2.1.1 The agent

1.2.1.1.1 Microbiological characteristics

S. agalactiae is a gram-positive facultative anaerobic bacterium classified as a group B streptococcus (GBS). It was first identified as a bovine mastitis-causing pathogen in 1887 (Nocard and Mollereau, 1887) and subsequently incriminated in human infections (Lancefield and Hare, 1935; Fry, 1938). Morphologically, *S. agalactiae* is diplococcal (a pair of cocci), approximately 2µm in diameter, non-spore forming and non-motile (Timoney et al., 1988). On culture, it is distinguished based on the production of an extracellular diffusible protein, which acts synergistically with the staphylococcal β haemolysin resulting in a zone of complete haemolysis in blood agar – the so-called CAMP reaction (named after the discoverers of the phenomenon: Christie, Atkins and Munch-Petersen) (Christie et al., 1944). Biochemically, the organism is catalase-negative, non-acid-fast, does not hydrolyse aesculin but hydrolyses sodium hippurate and typically yields grey to whitish-grey colonies when grown on sheep blood agar (Fallon, 1974; Koneman et al., 1988).

1.2.1.1.2 *S. agalactiae* strains

A variety of tools including serotyping, multi-locus sequence typing (MLST), randomly amplified polymorphic DNA (RAPD), ribotyping and pulsed-field gel electrophoresis (PFGE) have been employed to characterise *S. agalactiae* isolates derived from human and bovine cases (Jensen and Aarestrup, 1996; Martinez et al., 2000; Daignault et al., 2003; Duarte et al., 2005; Sukhnanand et al., 2005; Zadoks et al., 2011). Serotyping of *S. agalactiae* is based on its capsular polysaccharide antigens (CPS), of which 10 CPS serotypes have been described to date (Ia, Ib, II, III, IV, V, VI, VII, VIII, IX) each being antigenically and structurally unique (Doare and Heath, 2013). However, about 5-10% of *S. agalactiae* strains are considered non-serotypeable (Radtke, 2012). Serotypes II, III and non-typeable ones have been reported as being predominant among bovine *S. agalactiae* mastitis cases (Daignault et al., 2003; Duarte et al., 2004; Dogan et al., 2005). Amongst human strains, serotypes Ia, Ib, II, III and V are commonest (Doare and Heath, 2013). In Denmark, 58% of bovine and 39% of human strains were found to belong to serotype III, with 90% of bovine and 5% of human strains being lactose fermenters (c.f. Jensen and Aarestrup, 1996). Although a genetic relationship between bovine and human serotypes III could be implied, Bohnsack et al. (2004) showed that these serotypes were largely genetically distinct suggesting limited interspecies transmission.

MLST (based on housekeeping genes) grouping of *S. agalactiae* strains into sequence types (ST) has been observed not to strictly conform to capsular serotypes (Bisharat et al., 2004; Bohnsack et al., 2004; Brochet et al., 2006). The most prevalent human STs are ST 1, ST 17, ST 19 and ST 23 whereas ST 67 is the predominant type amongst bovine strains (Bisharat et al., 2004). However, several studies have noted that, despite STs 2, 17, 19 and 23 being significantly more common in humans, they are also recoverable from bovine populations (Bisharat et al., 2004; Bohnsack et al., 2004; Oliveira et al., 2006). In Denmark, MLST of 111 isolates from a 2009 BTM survey found the prevailing STs to be ST 1 (28%) and ST 23 (23%) (Zadoks et al., 2011). Notably, human isolates have been reported to exhibit greater diversity in STs than bovine isolates and further cluster separately from bovine isolates (Bisharat et al., 2004; Dogan et al., 2005). This genetic distinction has also been revealed by other genotyping methods such as RAPD, ribotyping and PFGE (Dogan et al., 2005; Duarte et al., 2005; Sukhnanand et al., 2005; Zadoks and Schukken, 2006). Notwithstanding this, Pereira et al. (2010) contend that genetic relatedness is not necessarily a prerequisite for *S. agalactiae* to cross the interspecies barrier.

1.2.1.2 The hosts

Humans and bovines are the primary species colonised by *S. agalactiae* (Keefe, 1997; Baker, 2000), although the agent has been implicated in illness in other hosts such as fish, camels, horses, dogs and cats (Yildirim et al., 2002a,b; Younan and Bornstein, 2007; Mian et al., 2009). In humans, *S. agalactiae* remains a leading cause of neonatal sepsis and meningitis in infants (Doare and Heath, 2013). In adults, it is associated with breast abscessation, (Rench and Baker, 1989) osteoarticular infections, pneumonia and urosepsis (Farley, 2001). Asymptomatic carriage may occur in the urogenital and gastrointestinal tracts as well as the skin (Van der Mee-Marquet et al., 2008) and is frequent in young adults (20-40%) and the elderly (22%) (Manning et al., 2004, 2008; Edwards and Baker, 2005). In cattle, *S. agalactiae* colonises the udder resulting primarily in subclinical mastitis (McDonald, 1977; Keefe, 1997). However, differences with respect to infection presentation in cows infected with human and bovine strains have been noted (Jensen, 1982). Whilst

experimental challenge of lactating quarters with human strains of *S. agalactiae* led to clinical mastitis on the one hand, on the other hand, bovine strains were observed to produce chronic subclinical infection. Moreover, human strains demonstrated a greater tendency to spontaneously clear (Jensen, 1982). Adherence abilities to the mammary gland tissue by the different strains are believed to determine their virulence (Jain, 1979). Infected cows typically exhibit high somatic cell counts (SCC) (up to 1 million cells/mL) and can shed up to 100 million bacteria/mL, with infected herds recording standard plate counts in the range of 20,000 to 100,000 colony-forming units (CFU)/mL (Keefe, 1997). Nonetheless, cows infected with human strains have been found to excrete lower levels of bacteria per mL compared to those infected with bovine strains (Jensen, 1982).

S. agalactiae has long been perceived as being an obligate pathogen of the udder, (McDonald, 1977; Keefe, 1997) but work by Manning et al. (2010) demonstrated that *S. agalactiae* ST 1 was recoverable from bovine faeces suggesting the possibility of transient rectal colonisation and thus a potential source[1] of infection for the udder. Considering that multiple organs in humans are prone to *S. agalactiae* infections whereas only the udder is known to be affected in cattle, as previously noted, the comparably low genetic heterogeneity detected in bovine isolates is justifiable (Zadoks et al., 2011).

1.2.1.3 The environment

In this context, the concept of environment is applied to denote the physical or biological sources of *S. agalactiae* and the mode by which the agent is conveyed to the susceptible hosts. Simply, the environment provides the necessary means by which the agent interacts with the host. In humans, the gastrointestinal tract is considered the main reservoir[2] of *S. agalactiae* and the source of vaginal colonisation in women (Doare and Heath, 2013). The pathogen has also been reported to be sexually transmissible (Manning et al., 2002). Infants become infected either through vertical transmission during birth (Doare and Heath, 2013) or from breastfeeding (Bingen et al., 1992). In cattle, the infected udder of heifers or cows is deemed the primary reservoir of infection (Keefe, 1997) although, as earlier alluded to, rectal colonisation may serve as a potential source of the organism (Manning et al., 2010). Notably, cross-suckling amongst co-housed, pre-weaned calves that are fed on infected raw milk may explain intramammary infections (IMI) with *S. agalactiae* in newly-calved heifers (McDougall et al., 2009; Petzer et al., 2013). Therefore, purchase of infected animals provides a conducive avenue for the introduction of infection into herds (Agger et al., 1994), with spread within the herd thought to occur during milking via the teat skin, milkers' hands, milking equipment and use of common udder cloths contaminated with milk from infected cows (Keefe, 1997). Based on work by Becker (1994), the survivability of *S. agalactiae* on various materials is as summarised below in Table 1:

[1]The source of a pathogen is the site from which it is transmitted to a susceptible host either directly or indirectly via an intermediary object (Baron, 1996).

[2]The reservoir of a pathogen is the site where it lives, metabolises and multiplies (Baron, 1996).

Table 1
Survival duration of *S. agalactiae* on various materials.

Material	Survival time
Hands and milkers' clothes	Up to 10 days
Skin of cows contaminated with *S. agalactiae* milk	About 14 days
Milk fat	14 – 21 days

Nevertheless, it is noteworthy that the agent is only reported to multiply within the udder (Keefe, 1997).

The possible mechanism of human-bovine transmissions has been investigated. Amongst humans, acquisition of bovine *S. agalactiae* following consumption of unpasteurised milk may potentially lead to throat infections (Brglize, 1981; Jensen and Aarestrup, 1996). In particular, STs 1 and 19 (found in both species) are believed to be orally acquired (Van der Mee-Marquet et al., 2008). Furthermore, a study by Manning et al. (2010) showed that *S. agalactiae* colonisation in humans was associated with increasing cattle exposure inciting speculation as to the possible role of bovine faecal shedding in human colonisation. Bovine udder infections with human *S. agalactiae* may arise as a result of contact with asymptomatic carriers of the pathogen (Jensen and Aarestrup, 1996; Andersen et al., 2003). In this regard, relief milkers, veterinarians and inseminators may facilitate transmission of the agent between herds (Edmondson, 2011). Figure 2 sums up the probable mechanisms by which *S. agalactiae* is introduced into a susceptible herd (either internally or externally) and subsequently spread within it.

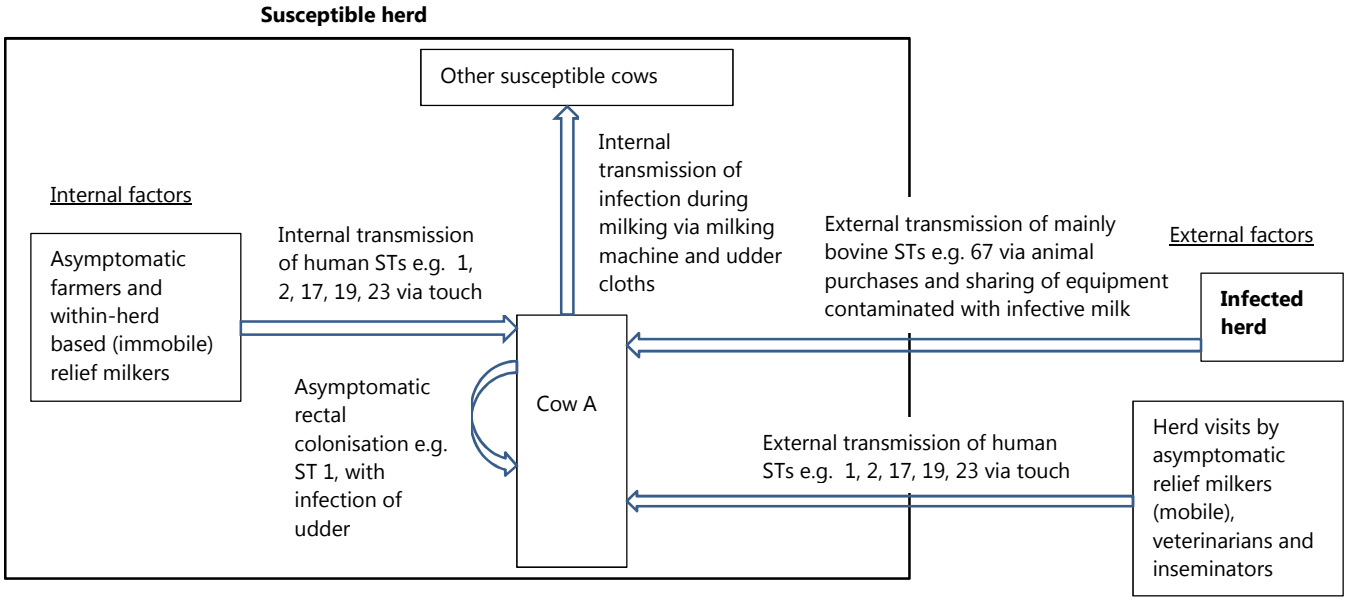

Fig. 2. Diagrammatic illustration of the between-and within-herd spread of *S. agalactiae*.

1.2.2 Impact of *S. agalactiae* infections

Allocation of resources for the detection and control of *S. agalactiae* in dairy herds is justified based on the following:

- Economic losses that are attributable to: (1) reduction in milk production – based on the finding that each increase in bulk tank milk somatic cell count (BTMSCC) of 100,000 cells/mL above a threshold of 200,000 cells/mL was associated with a 2% decrease in average herd production (Eberhert et al., 1982), Keefe et al. (1997) estimated the loss to be $3866 higher in infected herds compared to non-infected ones amongst dairy herds in Prince Edward Island. (2) Milk quality penalties associated with elevated BTMSCC and bacteria counts – these were valued at $550/year higher in infected herds than non-infected ones for the increase in BTMSCC and $78 per infected herd due to increased bacteria counts. (3) Reduction in milk quality resulting in prohibition to deliver milk for an extended period of time – which corresponded to $9084 for an average herd (Keefe et al., 1997).
- Zoonotic concerns. Anecdotally, human infections may result from consumption of unpasteurised milk (Brglize, 1981; Jensen and Aarestrup, 1996).

1.2.3 Herd-level detection of *S. agalactiae*

Diagnosis of *S. agalactiae* at the herd-level is based on screening of BTM samples. BTM testing is considered an inexpensive and rapid tool for evaluating milk quality and trouble-shooting herds with udder health problems (Jayarao and Wolfgang, 2003). Recovery of contagious mastitis pathogens (i.e. *Staphylococcus aureus*, *S. agalactiae* and *Mycoplasma* spp.) from the BTM serves as a suitable surrogate for IMI in at least one udder quarter in the herd (Jayarao and Wolfgang, 2003; Olde Riekerink et al., 2009). Owing to its contagiousness and subclinical presentation, control of *S. agalactiae* calls for early, rapid and accurate detection of infected herds (Keefe, 1997). Available BTM screening tests for *S. agalactiae* include the conventional bacteriological culture and recently, molecular-based techniques such as the polymerase chain reaction (PCR). Latex agglutination, enzyme-linked immunosorbent assay (ELISA), immunofluorescent assay (IFA) and slide coagglutination tests also hold promise. Their use is however undermined by the requirement of preliminary culture (Keefe, 1997).

1.2.3.1 Bacteriological culture

Culture of BTM has long been held as the reference method for herd diagnosis of *S. agalactiae* (Godkin and Leslie, 1993). A single culture of the BTM is regarded as having a high specificity (Sp) since the infected udder is considered the primary reservoir of infection and as such, isolation of the pathogen from the BTM is viewed a reflection of bacterial shedding by infected quarters in the herd (Bartlett et al., 1991). This argument is also supported by the fact that *S. agalactiae* was reported not to multiply in BTM except at temperatures over 27^{o}C (Gonzalez et al., 1986). Even so, Andersen et al. (2003) point out that the herd Sp of culture can be biased due to potential cross-contamination that can arise either during milk collection associated with residual milk from previously sampled infected herds or from processing of samples in the laboratory. Contrastingly, the sensitivity (Se) of a single BTM culture is low and highly variable amongst published studies, with estimates ranging from 20.5% (Godkin, 1989) to 78% (Keefe et al., 1997). This variability is ascribed to variations in culture protocols, within-herd prevalences, degree of bacterial shedding, which is related to the stage of infection and cyclicity in shedding patterns of infected glands (Gonzalez et al., 1986). In particular, with respect to the culture protocol, it is notable that there is not yet an established industry standard for BTM culturing (NMC, 1996). This is likely to bring about differences in the classification of positive and negative culture outcomes (case definition) thus impairing the comparison of test estimates across settings (Godkin

and Leslie, 1993). Additionally, the presence of growth-inhibitors in milk such as lactoferrin, lysozyme, immunoglobulins and lactoperoxidase may compromise the Se of culture (Rainard and Riollet, 2006). In an effort to improve culture Se, repeated BTM cultures within reasonable intervals accompanied by parallel interpretation of the test results has been advocated (Godkin and Leslie, 1993). By employing this strategy, Keefe et al. (1997) reported a combined protocol Se of 91% compared to the individual sensitivities range of 65% to 78%. Moreover, as more than 5% of milking quarters must be shedding *S. agalactiae* to enable its isolation in the BTM (Postle, 1968), it has been proposed that using larger milk inocula could increase Se of single cultures (Godkin, 1989; Andersen et al., 2003).

Besides its low Se, bacteriological culture is slow often requiring incubation periods of up to 72 h (NMC, 1999). These aspects necessitate the adoption of more sensitive, time-efficient diagnostic methods that possess potential for being rolled out routinely for *S. agalactiae* screening.

1.2.3.2 PCR

PCR methods developed for the diagnosis of mastitis-causing pathogens have been commended for their: (1) speed in execution (Koskinen et al., 2010), significantly reducing the duration of therapy and thus improving prognosis, (2) high sensitivity – demonstrating a capacity for the detection of both growth-inhibited and (3) objectivity and ease of automation (Martinez et al., 2001; Meiri-Bendek et al., 2002; Phuektes et al., 2003; Koskinen et al., 2009, 2010). Notwithstanding, a couple of demerits have also been noted: (1) as compared to culture, PCR methods possess a narrower scope, targeting only specific species for which they have been developed, (2) since their key target is DNA, non-viable cells can also be identified raising questions as to their clinical relevance (Schukken et al., 2010). In this regard, interpretation of the test outcomes should be performed taking into account the individual's clinical history (Koskinen et al., 2010). (3) The presence of milk Ca^{+2}, proteinases, fat and proteins can forbid the polymerase enzyme from accessing the target DNA, hence affecting the assays' Se (Wilson, 1997). Nonetheless, given the epidemiological characteristics of *S. agalactiae*, monitoring of the BTM for the pathogen by PCR has been suggested as a viable option (Phuektes et al., 2003).

As of 2008, a real-time PCR assay, the PathoProof Mastitis PCR (Finnzymes Oy, Espoo, Finland) has come into use exhibiting a potential for direct use in raw or preserved milk without the need for bacteriological culturing (Koskinen et al., 2009). Apart from *S. agalactiae*, 10 other mastitis-causing pathogens are detected i.e. *Staphylococcus aureus*, coagulase-negative *Staphylococci*, *Streptococcus dysgalactiae*, *Streptococcus uberis*, *Escherichia coli*, *Enterococcus* spp., *Klebsiella* spp., *Corynebacterium bovis*, *Serratia marcescens*, *Arcanobacterium pyogenes* and *Peptostreptococcus indolicus* (Koskinen et al., 2009). Being quantitative in nature, the assay records outcomes on a continuous scale referred to as the cycle threshold (Ct) values. The Ct value reflects the PCR cycle at which the fluorescence signal exceeds a set threshold level (Caraguel et al., 2011). The PathoProof Mastitis PCR assay comprises 40 cycles. Essentially, low Ct values signify high concentrations of DNA in the milk sample. Selection of an appropriate cut-off, upon which a sample is positively or negatively scored, is dependent on the relative cost of false negatives and false positives i.e. to either optimise Se or Sp respectively (Caraguel et al., 2011). Granted the relatively low shedding patterns of human *S. agalactiae* strains, (Jensen, 1982) their predominance in Danish dairy herds (Zadoks et al., 2011) may justify the PCR use in Denmark where bacteria concentrations in the BTM are expected to be low.

Moreover, as repeated testing of the BTM is recommended because of the cyclical shedding pattern for *S. agalactiae*, (Godkin and Leslie, 1993) given its higher Se (than culture) the number of screenings is expected to be lower, thereby ensuring cost savings.

1.2.4 Herd-level control of *S. agalactiae*

Dowdle (1998) defined disease control as the reduction in incidence, prevalence, morbidity and mortality of an infectious disease to a locally acceptable level. Here, the term is confined to incidence and prevalence reduction.

1.2.4.1 Reduction of incidence

To minimise the risk of new herd infections with *S. agalactiae* and subsequent spread within herds, a control programme should incorporate both between- and within-herd biosecurity measures.

1.2.4.1.1 Between-herd biosecurity

This refers to measures taken to prevent the risk of infection transmission between herds, and hence the introduction into susceptible ones (Barkema et al., 2009; Keefe, 2012). *S. agalactiae* may be brought into herds either by purchase of heifers/cows or sharing of relief milkers, inseminators and veterinarians amongst herds (Agger et al., 1994; Edmondson, 2011) (see Fig. 2).

As regards purchase of animals, preferably, herds should remain closed. However, in a bid to achieve rapid herd expansion or fulfil herd genetic improvement goals, preference may be given to purchase of animals as opposed to allowing internal herd growth (Keefe, 2012). This option carries with it a risk since the acquired animals may be infected. There is therefore need to adopt strategies that minimise the risk of transmission via this route. Barkema et al. (2009) offer guidelines that apply both to the herd of origin and the candidate animals. As pertaining to the source herd: (1) it should have a geometric mean BTMSCC of less than 200,000 cells/mL, (2) it should have records of individual cow SCC and (3) it ought to be free from *S. agalactiae* in the last 2 years. In Denmark, farmers can readily obtain this information from a public database, the *B*-register (see details under section 1.2.5.1.1). As relating to the specific animals, even though both heifers and cows could harbour *S. agalactiae*, heifers may pose less risk. In that case, purchase of pregnant non-lactating heifers should be regarded as an optimal choice. Nevertheless, if cows are purchased, they should have complete records of their entire lactation SCC and that, in the last lactation, the cow should not have SCC exceeding 200,000 cells/mL in any given test-day. In addition, three of the most recent SCC should be less than 100,000 cells/mL in the current lactation for the cow. The animals to be purchased should also be free of udder, teat and milk abnormalities. Prior to including the new purchases into the herd, acquired cows ought to be milked last until all their SCC tests register low SCC for three successive days.

Pertaining to the risk posed by herd personnel, Barkema et al. (2009) suggest that: (1) herds with a significant number of relief milkers should consider downsizing, (2) external personnel ought to be barred from handling cows in the parlour, (3) milking attendants should be educated on personal hygiene, biosecurity protocols and mastitis prevention and (4) provision of gloves, sanitary and hand-washing facilities should be prioritised. In

particular, Olde Riekerink et al. (2008) reported that gloved and disinfected hands had 75% and 98% respectively, lower bacteria loads than bare hands.

1.2.4.1.2 Within-herd biosecurity

For some herds, the risk of infection may not emanate from external sources but rather from internal ones i.e. farmers and within-herd based relief milkers (see Fig. 2). But regardless of the source, in the event that *S. agalactiae* is introduced into a herd, steps must be taken to minimise the risk of spread to uninfected cows. Measures that prevent both the internal introduction as well as subsequent within-herd spread of the infection fall under the umbrella of within-herd biosecurity (Barkema et al., 2009; Keefe, 2012). Internal introduction of infection may be prevented in a manner similar to the aforementioned: close observance of hygiene, hand gloving and disinfection.

For effective control of contagious mastitis pathogens, Neave et al. (1969) devised the five-point plan, which comprises: (1) adoption of sound husbandry and milking practices coupled with regular maintenance of the milking machine, (2) use of post-milking teat disinfection (PMTD), (3) prompt antimicrobial therapy of clinical cases during lactation, (4) culling of chronically infected cows and (5) blanket dry cow therapy (DCT) with antimicrobials. In order to reduce within-herd spread and hence the incidence of *S. agalactiae*, points 1 and 2 are key. Points 3 to 5 deal with prevalence reduction (see section 1.2.4.2).

In point 1, application of proper milking technique encompasses pre-milking udder hygiene, stimulation of milk let down and efficient milk removal (Radostits et al., 1994). Prior to milking, teats should be properly cleaned and dried to ensure good milking performance (Merrill et al., 1987) and to reduce the bacterial population, and thus avert new IMI (Galton et al., 1986). Cloth towels used for drying should be used on single cows and washed between uses. Maximum stimulation is suggested as an effective practice to ensure rapid milk flow and reduce teat-end stress. However, with adequately functioning milking equipment, there is insufficient evidence to support the requirement of manual massage (Radostits et al., 1994). During milk removal, efforts should be made to avoid liner slips and minimise machine stripping (NMC, 2009). It has been reported that a majority of IMI occur towards the end of milking (Spencer, 1989). Liner slips are common near the end of milking during which small droplets of contaminated milk may be propelled back against the end of the other teats, such that, after a sustained period of time, IMI may develop. Machine stripping is the act of exerting hand pressure on the milking unit at the end of milking for the purpose of removing extra milk. This practice may lead to liner slips and eventually new IMI (Radostits et al., 1994). Overmilking is discouraged to prevent teat end irritation that may predispose quarters to IMI. To further reduce the risk of spread between infected and uninfected herd mates, two approaches have been recommended: (1) adherence to a specific milking order with infection-free animals being milked first (Barkema et al., 2009) and (2) back-flushing of the milking unit to prevent exposure of uninfected cows to units contaminated by previously milked infected cows (Keefe, 2012).

The milking machine can influence new IMI in several ways (Radostits et al., 1994): (1) it may serve as a carrier of pathogens from one cow to the next, (2) it may form an efficient pathway of cross-infection within cows, (3) malfunctioning or improperly used machine may result in failure to relieve teat congestion

culminating in teat-end damage and possible IMI and (4) sudden loss of milking vacuum may thrust pathogens past the teat canal defences leading to IMI. Therefore, regular servicing, maintenance and evaluation of the machine according to the manufacturer's guidelines are recommended. In addition, regular replacement of liners, rubber and plastic parts together with broken and cracked inflations is necessary. Following each milking, the machine should be thoroughly washed and sanitised (NMC, 2009).

In point 2, PMTD remains an effective means to lower bacteria load on the teat skin and thus new IMI (NMC, 2007). Post-milking teat dips were shown to reduce the incidence of IMI with *S. agalactiae* by 48.1% to 71% (Pankey et al., 1983). The effectiveness of the procedure is dependent on the disinfectant establishing contact with the teat skin for at least 30 seconds and having good teat coverage when applied either as spray or dip (NMC, 2007).

1.2.4.2 Reduction of prevalence

Apart from incidence, the aim of a *S. agalactiae* control programme should be to eliminate[3] existing infections within a herd, with the ultimate goal of re-establishing the herd's free status. In order to achieve this, points 3 to 5 of the formerly mentioned five-point mastitis plan come into play.

Concerning point 3, *S. agalactiae* exhibits high antimicrobial susceptibility (Erskine et al., 2002; Makovec and Ruegg, 2003), although strain-specific differences have been observed, with bovine strains demonstrating a higher degree of susceptibility (Dogan et al., 2005). Often, a test and treatment approach (also known as partial blitz therapy) is implemented (Radostits et al., 1994). In a culture-and-treat protocol, the entire herd is cultured, after which all infected animals are treated at three-week intervals until all cows have had two consecutive negative cultures (Farnsworth et al., 2011). Approximately 5% to 10% of treated cases are refractory (Farnsworth et al., 2011). In those cases, culling (Point 4) is recommended to rid the herd of such potential reservoirs of infection. However, if the farmer elects to retain these chronic cows, arrangements should be made to ensure that they are milked last. The objective of instituting therapy during the dry period (point 5) is two-fold (Halasa et al., 2009a, b): (1) to eliminate infections present at the time of drying off and (2) to minimise the rate of new IMI during the dry period. Blanket DCT of all quarters of all cows is recommended as a standard practice. Nevertheless, existing legislation in some countries e.g. Nordic states that strongly favours judicious use of antimicrobials may only permit selective DCT (Katholm et al., 2012). Notably, a meta-analysis revealed that cows receiving blanket DCT had a 50% to 90% lower risk of new IMI compared to those not receiving blanket DCT (Robert et al., 2006). In Denmark, a 2008 survey involving 77 herds (33% of *B*-registered herds) revealed that, while 97% of the farmers admitted to exercising regular control of their milking equipment, only 75% and 74% of them practised PMTD and selective DCT respectively (Katholm, 2010a).

[3]Elimination is defined as the reduction of the prevalence of an infection to zero in a defined area (Dowdle, 1998).

1.2.5 *S. agalactiae* situation in Denmark and other countries

1.2.5.1 In Denmark

1.2.5.1.1 *S. agalactiae* surveillance, control schemes and accompanying legislative issues

Deliberate attempts at controlling *S. agalactiae* in the national dairy herd began during the period 1954 to 1958 based on a sample of 45 farms enrolled for an eradication[4] campaign of specific *Streptococci*. By 1958, of the initial 13 infected herds (29%), only 4 (9%) remained infected (Livoni, 1959). This success paved way for the issuance of the first legislative order on the control and monitoring of mastitis in 1962 (Anon., 1962). The directive provided for a voluntary control programme, in which farmers, especially those delivering milk to dairy factories, were encouraged to participate. The control efforts included hygienic and sanitary measures together with improved milking practices to minimise within-herd spread of *S. agalactiae*. Yet, it was not until 1966 when the first national screening of *S. agalactiae* in BTM came into being (Anon., 1966-1982). In particular, only 30.9% of the national herd was surveyed, with the number of participating herds increasing in the following surveys. In 1983, surveillance and control of *S. agalactiae* became mandatory (Anon., 1983). The provision required screening for *S. agalactiae* to be carried out on all BTM and individual cow milk samples. Infected farms received advice on improvement of parlour hygiene, maintenance of milking equipment functionality and methods to control mastitis causative factors. Treatment of infected cows was also instituted. All associated expenses i.e. procurement of cow milk and BTM samples, testing, treatment and provision of technical advice were defrayed by the state. Only for those herds deemed highly infectious and hence a threat to others, was the veterinary directorate mandated to impose restrictions on the sale of heifers and cows for live use as well as participation in animal shows.

In 1991, despite the mandatory surveillance of BTM samples remaining in force, some legislative changes were nonetheless effected (Anon., 1991). Collection and testing of cow milk samples, treatment of infected animals and advice on *S. agalactiae* control became elective. Additionally, all infected herds were banned from selling pregnant heifers and cows for live use and engaging in animal shows or other gatherings where milking was likely. As of 1999, farmers did not have to abide by the 1991 regulation requiring that they only use milking disinfectants approved by the veterinary department (Anon., 1999).

In 2005, the legislation required that a herd be designated as infected and hence entered into the *B*-register if either its positively identified BTM sample was confirmed in at least one of two subsequent tests or a submitted milk sample from one of the cows in the herd tested positive. A herd could redeem its status from the *B*-register if all its cow-level tests returned negative within the same day of testing or if four of its consecutively tested BTM samples (30 days apart) registered negative results. As regards the cow-level tests, any negatively-testing cows that had antibiotic therapy administered within four weeks prior to the testing would have to be subjected to further tests. It is notable that the 2005 directive led to the lifting of the ban on the sale of heifers and cows as it was argued that the risk of transmission by purchase was negligible; but participation in shows or gatherings where milking was likely remained forbidden. Owners of infected herds were henceforth obligated to disclose their status to willing buyers (Anon., 2005a, b).

[4]Eradication is defined as the complete removal of a disease pathogen from a population (Dowdle and Hopkins, 1998).

Prior to 1995, screening of BTM samples for *S. agalactiae* had been conducted at varying intervals (from quarterly to every second year). However, as from 1995, testing has been carried out on a yearly basis (Andersen et al., 2003). Even though bacteriological culture had been the conventional screening tool and the basis for *B*-registration up till 1st September 2011, BTM screening by the PathoProof Mastitis PCR assay has been in operation since 2009 (Jørgen Katholm, personal communication, 2013).

1.2.5.1.2 Historical trends in *S. agalactiae* frequency (1966 – 1999)

Estimates of the annual herd-level true prevalences and incidence risks of *S. agalactiae* during the period 1966 – 1999 are given in Table 2 and graphically displayed in Fig. 3 (see chapter 3b for further details). During the first voluntary phase of the control programme, the true prevalence declined from about 4% in 1966 to approximately 2% in 1981. With all herds becoming enrolled for the mandatory programme in 1983, the prevalence initially rose to 3.4% but then declined to 1% in 1989. With the reversion to the voluntary programme in 1991 (2nd phase), the true prevalence peaked in 1992 at 2.1%, but declined to settle at 1.3% in 1999. The True incidence remained at a level below 0.5% as from 1993.

Based on 2010 PCR data obtained from 34 Danish dairy herds participating in a voluntary control programme for *S. agalactiae*, the interquartile range for the within-herd prevalence was estimated to be 5% to 34% (Katholm, unpublished data).

Table 2

Estimated annual herd-level true prevalences and incidence risks of *S. agalactiae* in the population of Danish dairy herds during the period 1966 - 1999.

Year	[a]N (*)	[b]INF	[c]AP (95% [d]PCI)	[e]TP (95% PCI)	[f]POPr	[g]INC‡	[h]AI (95% PCI)	[i]TI (95% PCI)
1966	136731 (42250)	1352	3.20 (3.04 - 3.37)	4.12 (3.67 - 4.52)	-	-	-	-
1967	121124 (43968)	1363	3.10 (2.94 - 3.27)	3.98 (3.54 - 4.38)	-	-	-	-
1968	116380 (51207)	1485	2.90 (2.76 - 3.05)	3.71 (3.29 - 4.08)	-	-	-	-
1969	105944 (54667)	1476	2.70 (2.57 - 2.84)	3.44 (3.04 - 3.79)	-	-	-	-
1970	95782 (52680)	1317	2.50 (2.37 - 2.64)	3.17 (2.79 - 3.51)	-	-	-	-
1971	75867 (52500)	1365	2.60 (2.47 - 2.74)	3.30 (2.91 - 3.65)	-	-	-	-
1972	71571 (56040)	1401	2.50 (2.37 - 2.63)	3.17 (2.79 - 3.50)	-	-	-	-
1973	64498 (50760)	1269	2.50 (2.37 - 2.64)	3.17 (2.79 - 3.51)	-	-	-	-
1974	60581 (49192)	1279	2.60 (2.46 - 2.74)	3.30 (2.91 - 3.65)	-	-	-	-
1975	55852 (48200)	1205	2.50 (2.37 - 2.64)	3.17 (2.79 - 3.51)	-	-	-	-
1976	54207 (46130)	1061	2.30 (2.17 - 2.44)	2.90 (2.53 - 3.23)	-	-	-	-
1977	48614 (41857)	879	2.10 (1.97 - 2.24)	2.63 (2.27 - 2.95)	-	-	-	-
1979	39351 (34826)	801	2.30 (2.15 - 2.46)	2.90 (2.51 - 3.25)	-	-	-	-
1980	36373 (31895)	606	1.90 (1.76 - 2.06)	2.35 (2.00 - 2.68)	-	-	-	-
1981	35121 (32875)	526	1.60 (1.47 - 1.74)	1.95 (1.62 - 2.24)	-	-	-	-
1982	34235 (33643)	942	2.80 (2.63 - 2.98)	3.57 (3.15 - 3.97)	-	-	-	-
1983	33111	894	2.70 (2.53 - 2.88)	3.44 (3.02 - 3.83)	-	-	-	-
1984	31250	625	2.00 (1.85 - 2.16)	2.49 (2.13 - 2.82)	-	-	-	-
1985	28267	424	1.50 (1.37 - 1.65)	1.81 (1.49 - 2.11)	-	-	-	-
1986	26467	397	1.50 (1.36 - 1.65)	1.81 (1.49 - 2.11)	-	-	-	-
1987	24833	298	1.20 (1.07 - 1.34)	1.41 (1.10 - 1.69)	-	-	-	-
1988	22600	226	1.00 (0.88 - 1.14)	1.14 (0.85 - 1.40)	-	-	-	-
1989	22444	202	0.90 (0.78 - 1.03)	1.00 (0.72 - 1.26)	-	-	-	-
1990	20091	221	1.10 (0.97 - 1.25)	1.27 (0.97 - 1.56)	-	-	-	-
1991	19168	292	1.53 (1.36 - 1.71)	1.85 (1.50 - 2.18)	-	-	-	-
1992	17570	302	1.72 (1.54 - 1.92)	2.11 (1.74 - 2.46)	16963	182	1.12 (0.97 - 1.28)	0.63 (0.37 - 0.90)
1993	16127	241	1.50 (1.32 - 1.69)	1.81 (1.45 - 2.15)	15792	142	0.91 (0.78 - 1.06)	0.25 (0.03 - 0.52)
1994	15850	204	1.28 (1.12 - 1.45)	1.51 (1.18 - 1.82)	15129	139	0.92 (0.78 - 1.09)	0.38 (0.12 - 0.65)
1995	14370	131	0.92 (0.77 - 1.08)	1.02 (0.72 - 1.31)	14157	91	0.66 (0.56 - 0.80)	0.13 (0.01 - 0.35)
1996	13419	147	1.08 (0.92 - 1.26)	1.24 (0.92 - 1.55)	13262	97	0.74 (0.60 - 0.89)	0.41 (0.18 - 0.65)
1997	12141	112	0.93 (0.77 - 1.11)	1.04 (0.72 - 1.34)	11903	65	0.58 (0.48 - 0.71)	0.11 (0.01 - 0.32)
1998	11527	131	1.14 (0.96 - 1.34)	1.32 (0.98 - 1.66)	11372	76	0.67 (0.54 - 0.84)	0.32 (0.09 - 0.57)
1999	10060	113	1.13 (0.94 - 1.34)	1.31 (0.95 - 1.66)	9884	69	0.71 (0.57 - 0.88)	0.26 (0.04 - 0.54)

[a]Total dairy herd population; *No. tested; [b]No. infected; [c]Apparent prevalence; [d]Posterior credibility interval; [e]True prevalence; [f]Population at risk; [g]Incidence; [h]Apparent incidence risk; [i]True incidence risk.

AP; TP; AI; TI are median estimates and are expressed as percentages.

‡Incidence data only available as from 1992.

Data for 1978 were missing.

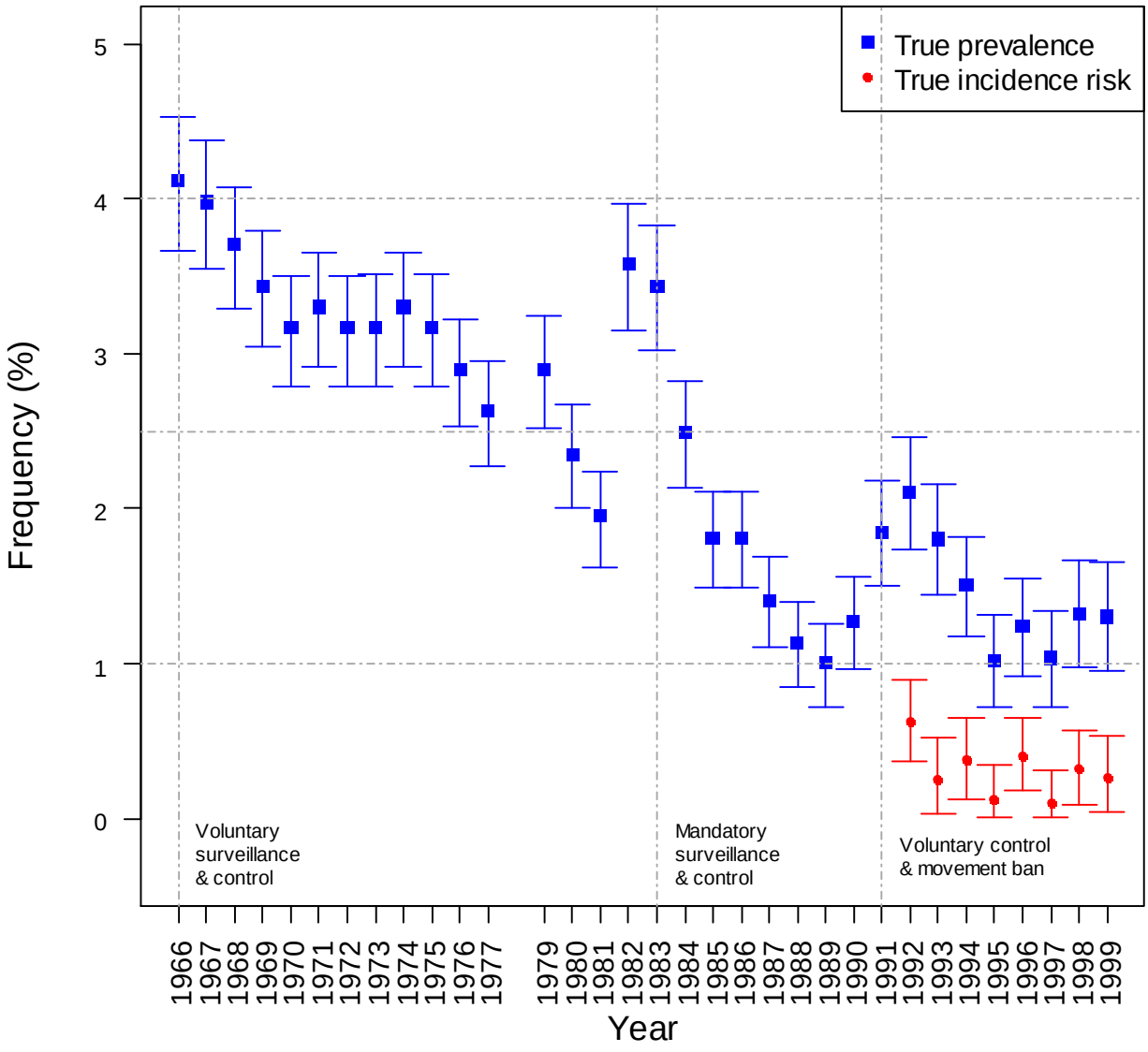

Fig. 3. Plot of the estimated annual herd-level true prevalences and incidence risks of *S. agalactiae* in the population of Danish dairy herds.

1.2.5.1.3 Danish cattle herd population

Descriptive statistics on the Danish dairy herd population during the period 2010 – 2013 are given in Table 3 (Danish Agriculture and Food Council, 2013). Notably, decreases in population sizes have been accompanied by increases in herd sizes and average milk yield per cow along with improvements in milk quality.

Table 3

Descriptive statistics on the populations of dairy and non-dairy herds, herd size, bulk tank milk somatic cell counts (BTMSCC/mL) and total bacterial counts (TBC expressed as CFU/mL) during the period 2010 – 2013.

Year	No. of dairy herds	Herd size	[a]Milk production (kg)	[b]BTMSCC/mL	[b]TBC (CFU/mL)	No. of non-dairy herds
2010	4138	126	8919	231500	9510	9331
2011	3953	132	9019	224700	8860	9325
2012	3887	138	-	221200	7800	8765
2013	3682	142	-	-	-	-

[a]Average milk production per cow in October in the given years.

[b]Expressed as a geometric mean.

1.2.5.2 In other countries

The herd-level prevalence of *S. agalactiae* varies widely across countries owing to differences in the extent of adoption of control measures as well as tests and testing protocols applied (NB: except where specified, the prevalences indicated relate to bacteriological culture). In Nordic countries where only selective DCT is permitted by law, the prevalence of *S. agalactiae* by PCR was 4.9%, 3.3% and 23.3% in Sweden, Norway and Faroe Islands respectively (Katholm, 2010b). In Finland, the pathogen has been detected at a level of 0.1% (Pitkälä et al., 2004). In the rest of Europe, the bacterium has been isolated at frequencies of 5.3%, 2.1%, 28.7% and 2.9% in Belgium (Piepers et al., 2007), Switzerland (Guélat-Brechbuehl et al., 2010), Germany (Tenhagen et al., 2006) and Czech Republic (Ryšánek et al., 2009) respectively. However in the Netherlands, a survey involving 49 dairy herds did not isolate *S. agalactiae* (Sampimon et al., 2009). In North America, *S. agalactiae* has been found at frequencies of 1.6%, 1.3% and 8.6% in Canada (Olde Riekerink et al., 2006, 2010; Francoz et al., 2012). Particularly, Olde Riekerink et al. (2010) reported that 72% and 96% of herds participating in a dairy herd improvement programme implemented blanket DCT and PMTD respectively. In the USA, the agent has been isolated at levels of 10% and 31% (Jayarao et al., 2004; Zadoks et al., 2004). In South America, *S. agalactiae* was reported to occur at frequencies of 11% in Uruguay, (Gianneechini et al., 2002) 39.7% in Brazil (Elias et al., 2012) and 42% in Colombia (Ceballos, 2013). In Australia, the prevalence of *S. agalactiae* by PCR was shown to be 26.2% (Phuektes et al., 2003).

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CHAPTER 2:

EVALUATION OF TWO HERD-LEVEL DIAGNOSTIC TESTS FOR *STREPTOCOCCUS AGALACTIAE* USING A LATENT CLASS APPROACH

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Abstract

Streptococcus agalactiae (*S. agalactiae*) mastitis persists as a significant economic problem for the dairy industry in many countries. In Denmark, the annual surveillance programme for this mastitis pathogen initially based only on bacteriological culture of bulk tank milk (BTM) samples, has recently incorporated the use of the real-time PathoProof Mastitis PCR assay with the goal of improving detection of infected herds. The objective of our study was to estimate the herd sensitivity (Se) and specificity (Sp) of both tests of BTM samples using latent class models in a Bayesian analysis while evaluating the effect of herd-level covariates on the Se and Sp of the tests. BTM samples were collected from all 4,258 Danish dairy herds in 2009 and screened for the presence of *S. agalactiae* using both tests.

The highest Se of PCR was realized at a cycle threshold (Ct) cut-off value of 40. At this cut-off, the Se of the PCR was significantly higher (95.2; 95% posterior credibility interval [PCI] [88.2; 99.8]) than that of bacteriological culture (68.0; 95% PCI [55.1; 90.0]). However, culture had higher Sp (99.7; 95% PCI [99.3; 100.0]) compared to PCR (98.8; 95% PCI [97.2; 99.9]). The accuracy of the tests was unaffected by the herd-level covariates. We propose that screenings of BTM samples for *S. agalactiae* be based on the PCR assay with Ct readings of <40 considered as positive. However, for higher Ct values, confirmation of PCR test positive herds by bacteriological culture is advisable especially when the between-herd prevalence of *S. agalactiae* is low.

Keywords: *Streptococcus agalactiae*; Latent class analysis; Diagnostic test; Herd sensitivity; Herd specificity; Cattle.

1. Introduction

Streptococcus agalactiae (*S. agalactiae*) is a highly contagious obligate pathogen of the bovine mammary gland which often causes subclinical mastitis in dairy cattle with attendant economic losses to the industry (Keefe, 1997b). The success of any control programme is largely dependent on the accuracy of screening tests which should limit the misclassification of test subjects (Christensen and Gardner, 2000). Since the inception of the Danish *S. agalactiae* control programme, herd screenings have been based on culture of bulk tank milk (BTM) (Andersen et al., 2003). Unlike environmental bacteria, such as the other streptococci, culture of the BTM for *S. agalactiae* has been justified by the obligatory nature of the pathogen whose finding in the pooled milk reliably indicates infection of the udder (Hogan and Smith, 1992). However, the herd sensitivity (Se) of bacteriological culture has been shown to be low and highly variable (Keefe, 1997b), often dependent on the protocol employed and the degree of bacterial shedding from infected cows, which in turn is related to the stage of infection (Gonzales et al., 1986).

Recently, a novel, rapid, real-time polymerase chain reaction (PCR) assay, the PathoProof Mastitis PCR (Finnzymes Oy, Espoo, Finland), has become commercially available. This assay has been considered as holding more promise than the conventional bacteriological culture given its capability in detecting both growth-inhibited and nonviable bacteria, and thus possesses an inherent potential for use in routine bovine mastitis testing programmes (Koskinen et al., 2009).

Although the performance of both BTM bacteriological culture and the PathoProof Mastitis PCR (at quarter-level) has been evaluated (Bartlett et al., 1991; Koskinen et al., 2009), this has only been based on imperfect reference standards which are subject to information and/or selection bias, and may therefore result in under or overestimation of the accuracy of the index test(s). In situations where neither a reasonable reference standard, nor a test(s) with known Se and Specificity (Sp) is existent, latent class models provide an invaluable option for the simultaneous estimation of Se and Sp of 2 or more tests without any assumption about the underlying true disease status of each subject. These models can be fit using maximum-likelihood procedures or Bayesian inference (Enøe et al., 2000). Three assumptions need to be considered when evaluating diagnostic tests using latent class models: (1) two or more populations with different prevalences are required, (2) the Se and Sp of the diagnostic tests should be the same across the populations, and (3) the tests should be conditionally independent given the disease status (Hui and Walter, 1980). Point 1 can be addressed by choosing stratifiers, which are independent of factors affecting Se and Sp (Nielsen et al., 2002). Point 2 requires that the "disease definition" or target condition be constant across populations. Constancy of the target condition is challenged when using quantitative tests such as real-time PCR, because the test response may be affected by differences in bacterial load, which affects the probability of test-positivity. A proposed solution has been use of latent class receiver-operating characteristics (ROC) curves (Wang et al., 2007). However, ROC analysis effectively forces the target condition to be constant, and thus masks differences in bacterial load which might result from specific covariates affecting the load. Therefore, use of quantitative tests requires that tests be evaluated at a defined cut-off based on the purpose of testing.

There is mounting evidence suggesting that Se and specificity (Sp) of diagnostic tests vary within populations of herds (Greiner and Gardner, 2000). Gonzales et al. (1986) found that the Se of culture of BTM varied based

on the within-herd prevalence of *S. agalactiae*. This prevalence is also expected to differ between large and small herds, organic versus conventional herds and between farms with and without automatic milking systems (AMS). Availability of such covariate information affords opportunities for the calculation of stratum-specific estimates of Se and Sp in non-homogeneous populations.

This study therefore aimed at estimating the herd Se and Sp of bacteriological culture and the PCR test using latent class models in a Bayesian analysis, while evaluating the effect of herd-level covariates on the Se and Sp of both tests. Furthermore, the effect of changing the target condition was explored. The findings from this study will be central to improving detection of infected herds to which control measures can be applied.

2. Materials and Methods

2.1. Sample collection

BTM samples derived from all 4,258 Danish dairy herds were collected between the 20th of October, 2009 and the 6th of January, 2010 through the mandatory milk quality surveillance scheme. Information on geographical location of the herds, use of AMS, herd size and the type of production (organic or conventional), were obtained from the Danish Cattle Database. Sampling was conducted by the BTM truck drivers during milk collection, after which samples were stored on ice. Within 24 hours, they were sent to Eurofins Laboratory, Holstebro, Denmark, for processing. At the laboratory, samples were screened by bacteriological culture and the PathoProof Mastitis PCR.

2.2. Target condition

The target condition in this study was a BTM sample containing *S. agalactiae* or parts of it. Thus, any concentration of bacteria in the sample was considered a case, irrespective of whether the tests detected the bacteria or not.

2.3. Bacteriological culture

Each BTM sample was cultured following the National Mastitis Council standards. A 120 µl of milk inoculum was mixed with 9 ml of selective agar in a Petri dish containing 5% sterile calf blood, 1% wt/vol aesculin supplied with neomycin sulphate and Polymyxin B, sodium fusidate and *Staphylococcus aureus* β-toxin. This mixture was incubated for 18 to 24 hrs at 37°c. Any colonies showing β-haemolytic activity were counted on each plate following which one of the colonies was selected and recultured on 5% bovine blood agar with the *S. aureus* β-toxin to elicit the characteristic CAMP reaction. Isolates that were positive in the CAMP as well as in a Lancefield group B latex agglutination test were identified as *S. agalactiae*.

2.4. PathoProof Mastitis PCR

The PCR reactions were run using reagents and protocol instructions as described in the PathoProof Mastitis PCR manual (Finnzymes Oy). Briefly, 350 µl of milk was used as the starting volume for DNA extraction. The extraction protocol involved an enzymatic lysis step disrupting the somatic cells present in mastitic milk, a centrifugation step, an additional lysis step involving the disruption of the bacterial cell walls and a magnetic bead-based DNA purification and elution step. Cycle threshold (Ct) values were recorded for each sample. Notably, the assay's thermal cycling protocol involved 40 cycles; generally, the higher the CFUs in milk the

lower the resulting Ct value. For statistical analysis, 5 different Ct cut-off values of the PCR test were selected i.e. <31, <33, <35, <37 and <40. The cut-off value yielding the highest Se of PCR was selected for subsequent analyses.

2.5. Population classification

Data were aggregated by geographical location into 4 populations of herds with different densities comprising: the eastern Danish islands: Bornholm, Zealand and Funen (population 1); South Jutland (population 2); Mid-Jutland (population 3) and North Jutland (population 4). These populations were assumed to have different between-herd prevalences and hence formed the basis for the estimation of the herd Se and Sp of both tests.

2.6. Statistical model

We assumed that the true between-herd prevalence of *S. agalactiae* differed across the 4 regions. Additionally, constancy of Se and Sp of each of the 2 diagnostic tests was assumed across the populations. But in order to allow for separate estimates between large and small herds, organic versus conventional herds and farms with and without AMS, each population was stratified by each of the aforementioned herd-level covariates. Both tests were assumed to be conditionally independent given the herd infection status since they employ different techniques to detect the agent (Branscum et al., 2005). Therefore, detection of bacteria by either of the tests conditional on the infection status would be a function of their respective sensitivities. This issue is further elucidated in the discussion.

Counts (O_p) of the different test combinations e.g. POS/POS, POS/NEG, were assumed to follow a multinomial distribution: $O_p|Se_{ij},Sp_{ij},P_k \sim$ multinom – multinomial ($Prob_k$, n_k) for population k, test i in stratum j. $Prob_k$ is a vector of probabilities of observing the individual combinations of test results for the kth population. The probabilities are specified using the Se and Sp of the tests and the prevalence (P) of each population:

$$\mathbf{Prob}_k = \begin{pmatrix} \Pr(T_1^+T_2^+) \\ \Pr(T_1^+T_2^-) \\ \Pr(T_1^-T_2^+) \\ \Pr(T_1^-T_2^-) \end{pmatrix} = \begin{pmatrix} \Pr(T_1^+T_2^+|D^+)\Pr(D^+) + \Pr(T_1^+T_2^+|D^+)\Pr(D^-) \\ \Pr(T_1^+T_2^-|D^+)\Pr(D^+) + \Pr(T_1^+T_2^-|D^+)\Pr(D^-) \\ \Pr(T_1^-T_2^+|D^+)\Pr(D^+) + \Pr(T_1^-T_2^+|D^+)\Pr(D^-) \\ \Pr(T_1^-T_2^-|D^+)\Pr(D^+) + \Pr(T_1^-T_2^-|D^+)\Pr(D^-) \end{pmatrix}$$

$$= \begin{pmatrix} \mathrm{Se}_{1j}\mathrm{Se}_{2j}p_k + (1-\mathrm{Sp}_{1j})(1-\mathrm{Sp}_{2j})(1-p_k) \\ \mathrm{Se}_{1j}(1-\mathrm{Se}_{2j})p_k + (1-\mathrm{Sp}_{1j})\mathrm{Sp}_{2j}(1-p_k) \\ (1-\mathrm{Se}_{1j})\mathrm{Se}_{2j}p_k + \mathrm{Sp}_{1j}(1-\mathrm{Sp}_{2j})(1-p_k) \\ (1-\mathrm{Se}_{1j})(1-\mathrm{Se}_{2j})p_k + \mathrm{Sp}_{1j}\mathrm{Sp}_{2j}(1-p_k) \end{pmatrix}$$

where Se_{1j} and Se_{2j} are the Se of bacteriological culture and PCR respectively in stratum j. The same applies for Sp.

Therefore, for each stratifier the resulting 8 populations giving a total of 24 degrees of freedom were sufficient to estimate 16 parameters (stratum-specific Se and Sp estimates for each of the tests and 8 prevalence

estimates corresponding to the 8 populations). A Bayesian model implemented in OpenBUGS version 3.2.1 rev 781 (Thomas et al., 2006) was used to estimate the test parameters and population prevalences. Non-informative priors (beta(1, 1)) were used to fit the models since no prior information for the considered target condition was available.

We ran 20,000 iterations of the models with the first 10,000 discarded as the burn-in phase. Convergence of the Markov Chain Monte Carlo (MCMC) chain was assessed by visual inspection of the time-series plots of selected variables as well as the Gelman-Rubin diagnostic plots using two sample chains with different initial values. Hypotheses for the differences between stratum-specific test parameter estimates were evaluated based on a Bayesian posterior probability (POPR), the frequentist *P*-value analogue. Additionally, a separate model ignoring differences in test characteristics between stratifiers was constructed. The resulting nested models were compared using the Deviance Information Criterion statistic (DIC) (the smaller the value the better the fit). We also computed the Differential Positive Rate (DPR), which indicates the cut-off value at which Se and Sp are maximized simultaneously, as: DPR = (Se + Sp) – 100.

3. Results

The median herd size of 120 lactating cows (range 1 -1227) formed the basis for classifying herds into the *small* and *large* categories. Table 1 displays the cross-tabulated counts of the dichotomous outcome of the two tests. There were 530 (12.4%), 1,332 (31.3%), 1,406 (33.0%) and 990 (23.3%) herds included in populations 1, 2, 3 and 4 respectively. Of the farms, 22.3% had AMS and 9.3% were organic.

The stratum-specific estimates of Se and Sp of both tests at one of the PCR cut-off values (<40, which corresponds to the highest Se of PCR) are displayed in Table 2. There were no demonstrable significant differences between the stratum-specific estimates as indicated by the POPR value. Furthermore, the DIC estimate for the null model (Table 3) i.e. the model ignoring differences in test estimates between stratifiers, was considerably smaller than for the full model clearly in support of the simpler one. Thus, the results from the simpler model were used for subsequent analyses.

The Se of bacteriological culture decreased with increasing PCR cut-off values (Table 3). At a cut-off value of <40, the Se and Sp values of the PCR test were maximized (Se, 95.2; Sp, 98.8; DPR = 94). On the other hand, at the same cut-off, the culture test estimates were minimized (Se, 68.0; Sp, 99.7; DPR = 67.6).

In Fig. 1 the population-specific between-herd prevalences are plotted against each of the PCR cut-off values. For each population the prevalence increased with increasing Ct values. At the cut-off value of <40, the posterior mean prevalences were 4.3%, 6.3%, 6.4% and 8.4% for populations 1, 2, 3 and 4 respectively.

Table 1

Cross-tabulated results for bacteriological culture and real-time PCR by stratum based on PCR cut-off <40

Stratum	Test outcome (Culture / PCR)				Total (%)
	POS/POS	POS[a]/NEG[b]	NEG/POS	NEG/NEG	
Population					
1	15	3	10	502	530 (12.4)
2	53	7	40	1232	1332 (31.3)
3	56	7	45	1298	1406 (33.0)
4	54	3	37	896	990 (23.3)
Herd size					
Small (≤120)	54	5	55	2005	2119 (49.8)
Large (>120)	124	15	77	1923	2139 (50.2)
AMS[c]					
Yes	48	4	33	863	948 (22.3)
No	130	16	99	3065	3310 (77.7)
Production type					
Conventional	172	19	131	3538	3860 (90.7)
Organic	6	1	1	390	398 (9.3)

[a]Positive; [b]Negative; [c]Automatic milking system

Table 2

The Deviance Information Criterion (DIC), stratum-specific estimates of sensitivity and specificity of bulk tank bacteriological culture and PCR tests and a significance value for the difference between the stratum estimates at PCR cut-off <40

Test parameter [a]	Herd size		POPR[d] value	DIC
	Small (95% PCI[c])	Large (95% PCI)		
Se_{CUL}[b]	63.4 (44.9; 91.6)	73.9 (58.4; 96.5)	0.25	
Sp_{CUL}	99.8 (99.5; 100.0)	99.5 (99.0; 100.0)	0.80	123.4
Se_{PCR}	94.5 (84.9; 99.8)	94.0 (86.0; 99.7)	0.54	
Sp_{PCR}	98.8 (97.4; 99.9)	98.4 (96.1; 99.9)	0.61	

Test parameter	AMS		POPR value	DIC
	Yes (95% PCI)	No (95% PCI)		
Se_{CUL}	74.8 (54.1; 98.0)	66.9 (53.6; 90.3)	0.70	
Sp_{CUL}	99.7 (99.0; 100.0)	99.7 (99.3; 100.0)	0.51	120.8
Se_{PCR}	94.1 (84.6; 99.7)	94.3 (86.2; 99.7)	0.50	
Sp_{PCR}	98.0 (95.8; 99.9)	98.9 (97.2; 100.0)	0.27	

Test parameter	Production type		POPR	DIC
	Conventional (95% PCI)	Organic (95% PCI)		
Se_{CUL}	66.3 (54.3; 87.5)	78.7 (42.1; 99.1)	0.23	
Sp_{CUL}	99.7 (99.3; 100.0)	99.6 (98.7; 100.0)	0.48	99.8
Se_{PCR}	95.1 (88.0; 99.8)	78.6 (41.9; 99.1)	0.87	
Sp_{PCR}	98.8 (97.0; 99.9)	99.6 (98.7; 100.0)	0.19	

[a]Mean estimates.

[b]Culture.

[c]Posterior credibility interval.

[d]Bayesian posterior probability.

Table 3

The Differential positive rate (DPR), DIC and pooled estimates of sensitivity and specificity of bulk tank bacteriological culture and PCR tests at various PCR cut-off values

PCR Cut-off value	Test Parameter	Estimate (95% PCI)	[a]DPR_{CUL}	DPR_{PCR}	DIC
	Se_{CUL}	68.0 (55.1; 90.0)			
<40	Sp_{CUL}	99.7 (99.3; 100.0)	67.6	94.0	71.0
	Se_{PCR}	95.2 (88.2; 99.8)			
	Sp_{PCR}	98.8 (97.2; 99.9)			
	Se_{CUL}	76.3 (61.0; 97.3)			
<37	Sp_{CUL}	99.6 (99.1; 100.0)	76.0	88.9	70.7
	Se_{PCR}	90.0 (80.7; 99.2)			
	Sp_{PCR}	98.9 (97.7; 99.9)			
	Se_{CUL}	80.1 (66.3; 97.9)			
<35	Sp_{CUL}	99.5 (98.9; 100.0)	79.6	88.0	69.1
	Se_{PCR}	88.8 (77.8; 99.3)			
	Sp_{PCR}	99.2 (98.2; 100.0)			
	Se_{CUL}	83.6 (71.3; 98.1)			
<33	Sp_{CUL}	99.2 (98.5; 99.9)	82.8	86.0	69.3
	Se_{PCR}	86.5 (71.7; 99.2)			
	Sp_{PCR}	99.4 (98.7; 100.0)			
	Se_{CUL}	91.0 (80.5; 99.5)			
<31	Sp_{CUL}	99.0 (97.9; 99.9)	89.9	74.8	68.1
	Se_{PCR}	75.1 (56.6; 97.4)			
	Sp_{PCR}	99.7 (99.3; 100.0)			

[a]DPR: (Se + Sp) – 100

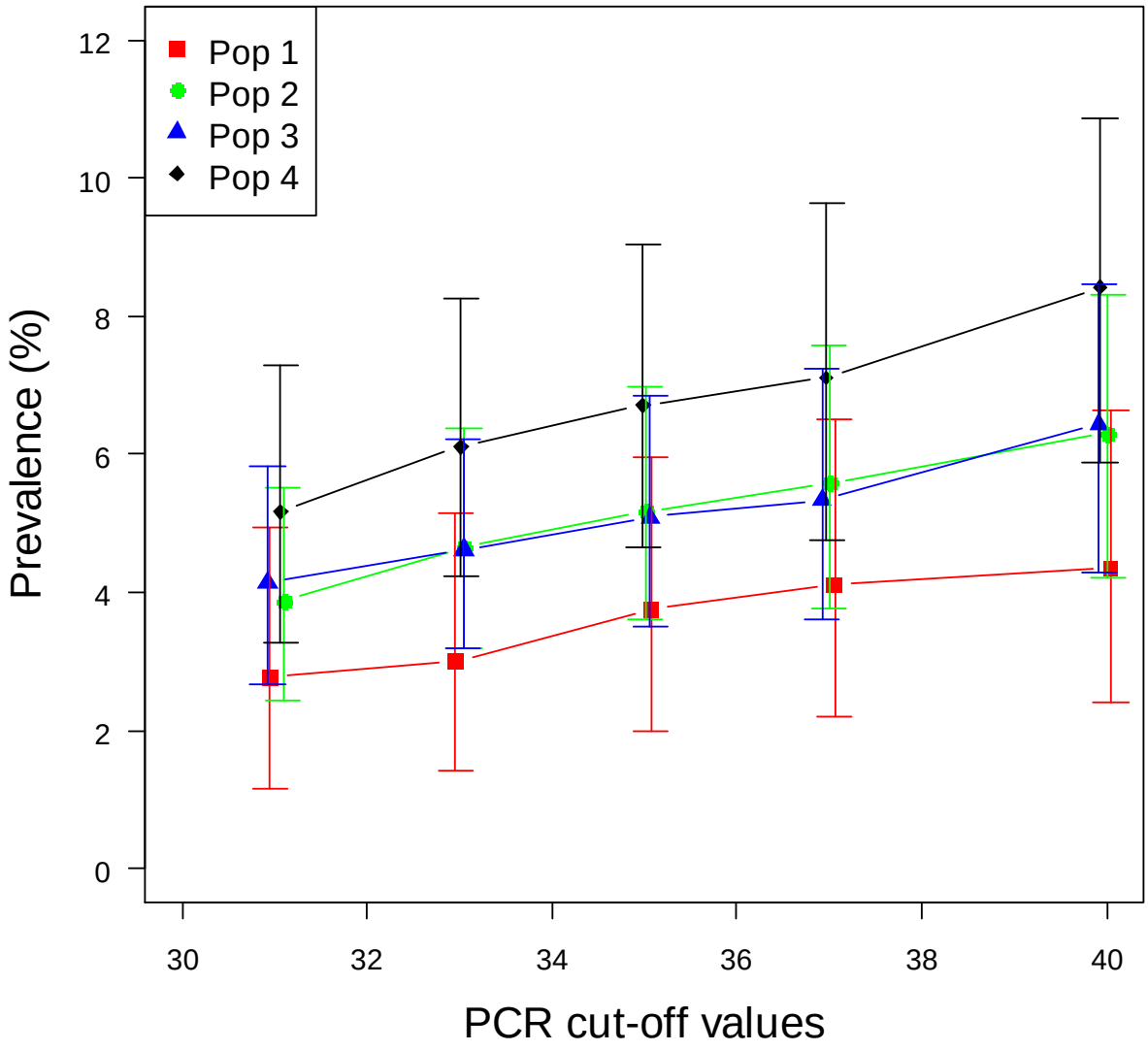

Fig. 1. A plot of the posterior mean prevalence estimates for the four populations at different PCR cut-off values.

4. Discussion

We have used a Bayesian framework to estimate the Se and Sp of both BTM bacteriological culture and PCR tests at the highest cut-off value of the PCR. To our knowledge, this is the first study to evaluate the performance of the PathoProof Mastitis PCR as a herd test. The analysis demonstrated that the PCR assay had higher Se but lower Sp than bacteriological culture. Although a PCR Ct value of 37 has been recommended as an appropriate cut-off for scoring reactions either positive or negative (Koskinen et al., 2009), this study has shown that a Ct value of <40 affords estimates of Se and Sp of the real-time PCR test yielding the highest DPR. A test with a high Se and reasonable Sp is desirable in a control programme in order to ensure that as many of the truly infected herds are detected with only a tiny fraction of those truly uninfected being misclassified as positive.

Even though the Sp of culture of *S. agalactiae* from BTM has been shown to be consistently high, its Se has been highly variable amongst published studies with estimates ranging from 20.5% (Godkin, 1989) to 78% (Keefe, 1997a). In those studies, estimation of culture test characteristics was based on the assumption of an existing perfect reference standard. In order to minimize bias in the test estimates while concomitantly ensuring internal as well as external validity (generalisability), we have employed latent class analysis of data derived from all the dairy herds in Denmark.

Greiner and Gardner (2000), contend that in non-homogeneous populations stratum-specific estimates of Se and Sp have greater diagnostic utility than crude (pooled) estimates. Although we allowed Se and Sp of both bacteriological culture and PCR to vary between strata, the resulting estimates were not statistically significantly different from each other (though some differences might exist). Thus, pooled estimates were computed. This implies that within a specific population, for either of the tests its ability to detect the herd infection status is the same regardless of the size of the individual herds, their production type or AMS status.

The between-herd prevalence of *S. agalactiae* was higher with higher cut-off values of PCR. This phenomenon can be explained as follows: at the lowest Ct value (<31), primarily herds with high colony forming units (CFUs) in their BTM would be included in the target condition. As the cut-off is further raised, an increasing number of herds with low CFUs would be added to the existing pool of herds with the target condition such that at the highest cut-off (<40), the target condition would constitute the entire spectrum of infection i.e. from the very heavily infected herds (high CFUs in BTM) to the very lightly infected (low CFUs in BTM), however, with a preponderance of lightly infected herds. This changing target condition has implications on the interpretation of Se and Sp estimates of both tests. Bacteriological culture Se was highest at the lowest cut-off (<31) whereas the PCR Se was lowest at the same cut-off value. This suggests that culture is superior to the PCR assay in detecting heavily infected herds. However, the probability of misclassifying non-infected herds is lower for PCR given its higher Sp at this cut-off. At the highest cut-off, which comprises mainly of lightly infected herds, the PCR assay outperforms culture at their detection. Nevertheless, considering the PCR lower Sp, it becomes necessary to confirm its positives by bacteriological culture. Andersen et al. (2003) propose that with declining pathogen concentrations in the BTM, it might be necessary to increase the amount of milk cultured in order to improve the Se of culture. The findings from this study therefore illustrate that the usefulness of either of these tests is dependent on the target condition (level of CFU in BTM) under consideration.

The uncertainty associated with the Se estimates of both tests is a reflection of the varying number of truly infected herds for each assay at different cut-off values that are used in the tests Se estimation. At the highest cut-off value of the PCR assay (<40), bacteriological culture had the largest uncertainty around its Se estimate whereas PCR had the smallest. At the lowest cut-off (<31), the reverse was true. Toft et al. (2005) demonstrated that the precision of the estimates of Se and Sp increased with greater difference in the prevalences amongst the populations studied. As earlier mentioned, the uncertainty associated with the Se estimates of both tests at different cut-offs was a consequence of the changing target condition.

The assumption of conditional independence of both tests is supported by 2 key arguments: (1) a difference in their detection techniques (bacteriological culture relies on the isolation of the pathogen whereas PCR targets the pathogen's DNA) and (2) the fact that the PathoProof Mastitis PCR assay is performed directly from raw milk, without the need for bacteriological culturing, unlike previous PCR-based mastitis tests (Koskinen et al., 2009). Therefore, amongst known infected herds with *S. agalactiae*, the probability of a positive result to PCR will be the same in BTM samples that test negative to bacteriological culture as it will be in those that test positive to culture. A similar interpretation applies to non-infected herds. Consequently, if the true herd infection status is known, knowing one test result will not change our belief of the result of the other test and as such, the test results can be considered as conditionally independent given the infection status of the herd.

As previously noted, screening of BTM samples for *S. agalactiae* is justified by its obligatory nature such that its presence in BTM is indicative of infected udder quarters (Hogan and Smith, 1992). In light of this fact, the tests' estimates obtained in this study should be considered as applicable to only *S. agalactiae* and thus not by any means extendable to other mastitis pathogens, which apart from the udder may have an environmental source. For these pathogens, environmental contamination of BTM would lead to false positive test results that would bias Sp estimates of the index tests. For this reason, quarter-level testing may arguably be the only reliable way to determine the infection status of the herd (Cousins, 1972). As a means to validate the belief that the number of *S. agalactiae* in milk is a function of the number of infected quarters shedding the organism (Keefe, 1997b), BTM PCR Ct values could be assessed against the within-herd prevalence of the pathogen. For a herd in which this prevalence is high, its corresponding Ct value would be expected to be low. However, this computation was not possible in the present study owing to a lack of within-herd prevalence data.

5. Conclusion

Using latent class analysis we have estimated the Se and Sp of both the conventional bacteriological culture and the real-time PCR assay. The real-time PCR has been shown to have a higher Se but lower Sp than the culture test. Consequently, screenings of bulk tank milk samples for *S. agalactiae* should rely on the PCR assay with Ct readings of <40 considered as positive. However, for higher Ct values, confirmation of PCR test positive herds by bacteriological culture is advisable especially when the between-herd prevalence of *S. agalactiae* is low.

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CHAPTER 3a:

ANNUAL INCIDENCE, PREVALENCE AND TRANSMISSION CHARACTERISTICS OF *STREPTOCOCCUS AGALACTIAE* IN DANISH DAIRY HERDS

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Abstract

Contagious mastitis pathogens continue to pose an economic threat to the dairy industry. An understanding of their frequency and transmission dynamics is central to evaluating the effectiveness of control programmes. The objectives of this study were twofold: (1) to estimate the annual herd-level incidence rates and apparent prevalences of *Streptococcus agalactiae* (*S. agalactiae*) in the population of Danish dairy cattle herds over a 10-year period from 2000 to 2009 inclusive and (2) to estimate the herd-level entry and exit rates (demographic parameters), the transmission parameter, β, and recovery rate for *S. agalactiae* infection.

Data covering the specified period, on bacteriological culture of all bulk tank milk samples collected annually as part of the mandatory Danish *S. agalactiae* surveillance scheme, were extracted from the Danish Cattle Database and subsequently analysed. There was an increasing trend in both the incidence and prevalence of *S. agalactiae* over the study period. Per 100 herd-years the value of β was 54.1 (95% confidence interval [CI] 46.0-63.7); entry rate 0.3 (95% CI 0.2 – 0.4); infection-related exit rate 7.1 (95% CI 5.6 – 8.9); non-infection related exit rate 9.2 (95% CI 7.4 – 11.5) and recovery rate 40.0 (95% CI 36.8 – 43.5). This study demonstrates a need to tighten the current controls against *S. agalactiae* in order to lower its incidence.

Key words: *Streptococcus agalactiae*; Incidence; Prevalence; Parameters; Transmission; Cattle

1. Introduction

Streptococcus agalactiae (*S. agalactiae*) is a contagious obligate pathogen of the bovine mammary gland, which primarily causes subclinical mastitis. Subclinical mastitis causes a substantial economic impact to dairy producers owing to production losses and its detrimental effects on milk quality (Keefe, 1997). Transmission of the pathogen within dairy herds may occur during milking via milkers' hands, liners and use of common udder cloths contaminated with milk from infected cows (Keefe, 1997). Hiring of relief milkers and purchase of cows or heifers have been associated with the spread of infection between herds (Agger et al., 1994; Edmondson, 2011).

In Denmark, a surveillance and control programme for *S. agalactiae* has been in place for decades. This was initiated against a backdrop of worrisome infection prevalences in the early 1950s (c.f. Andersen et al., 2003). The control programme was compulsory until 1988 (though the mandatory surveillance carried on) and entailed the identification of infected cows by bacteriological culture of quarter milk samples with subsequent treatment or culling. These measures were supplemented with improved milking practices and hygienic measures to control within-herd spread of infection. Afterwards, there was a switch to a voluntary programme but still with a prohibition to sell cows and pregnant heifers from herds declared to be infected (Andersen et al., 2003). These concerted efforts saw a drastic decline in the herd-level prevalence of *S. agalactiae* from 30-40% in 1950 (Anonymous, 1981) to about 2 % in 1992, with a 1-2% annual incidence of herd infections (Anonymous, 1980-1992). However, since 2000 the proportion of infected herds has been rising steadily. Monitoring of infection incidence and prevalence is central to evaluating the performance of control programmes (Neave et al., 1966).

Given the prohibitive costs and impracticalities of assessing the effectiveness of control measures in field trials, mathematical models have increasingly been employed to play this role (Zadoks et al., 2002; White et al., 2006). Furthermore, these models provide unique opportunities for the description of the dynamics of mastitis transmission (Barlow et al., 2009). The within-herd transmission dynamics of mastitis pathogens, in particular, *Streptococcus uberis* and *Staphylococcus aureus* have previously been investigated (White et al., 2001, 2006; Zadoks et al., 2001, 2002). However, to date and to the best of our knowledge, there are no published studies describing the between-herd transmission dynamics of *S. agalactiae*. An often used parameter to quantify transmission is the transmission rate, β which is defined as the average number of secondary infections caused by one infectious individual per unit of time (Keeling and Rohani, 2008). When exploration of the long-term persistence of a pathogen in a population is of interest, it is necessary to take into consideration demographic changes (entries and exits) that occur in the population over time. Knowledge of this transmission characteristic and demographic parameters can be used in modelling the effectiveness of control strategies against *S. agalactiae* and in the estimation of the basic reproductive ratio, R_o (the average number of secondary infections caused by one infectious individual, throughout its infectious period, in an entirely susceptible population (Keeling and Rohani, 2008)), which reflects a pathogen's potential for spread.

The objectives of this study were: (1) to estimate the annual herd-level incidence rates and apparent prevalences of *S. agalactiae* in the population of Danish dairy cattle herds over a 10-year period from 2000 to

2009 inclusive and (2) to estimate the herd-level entry and exit rates (demographic parameters), the transmission parameter, β, and recovery rate for *S. agalactiae* infection.

2. Materials and Methods

2.1. Data

Data covering the period 1998 to 2009 inclusive, on bacteriological culture of all bulk tank milk (BTM) samples collected as part of the mandatory Danish *S. agalactiae* surveillance scheme, were extracted from the Danish Cattle Database. The scheme involves an annual collection of BTM samples by truck drivers during milk collection, after which samples are stored on ice. Within 24 hours, they are sent to Eurofins Laboratory, Holstebro, Denmark, for processing. Bacteriological culture of the samples follows the National Mastitis Council (1999) standards, but briefly: for each BTM sample, a 120 µl of milk inoculum is mixed with 9 ml of selective agar in a Petri dish containing 5% sterile calf blood, 1% wt/vol aesculin supplied with neomycin sulphate and Polymyxin B, sodium fusidate and *Staphylococcus aureus* β-toxin. This mixture is incubated for 18 to 24 hrs at 37^{o}c. Any colonies showing β-haemolytic activity are counted on each plate, following which one of the colonies is selected and recultured on 5% bovine blood agar with the *S. aureus* β-toxin to elicit the characteristic CAMP reaction (named after the discoverers of the phenomenon: Christie, Atkins and Munch-Petersen) (Christie et al., 1944). Isolates that are positive in the CAMP as well as in a Lancefield group B latex agglutination test are identified as *S. agalactiae*. Notably, the sensitivity and specificity of the BTM bacteriological culture have been estimated to be 68.0% and 99.7% respectively (Mweu et al., 2012).

For some herds in certain years, particularly if the first screening result was positive, upon a farmer's request repeat testing was carried out. However, to ensure consistency with the rest of the data the first observation for any given herd in a given year was used to define the status of the herd. Any missing observations for herds over the 10-year period were considered to arise from either: (1) an omission from surveillance or contamination of samples prohibiting bacteriological culturing or (2) an exit from the population of herds which could be a permanent or temporary cessation in milk production followed by a re-entry (resumption in production). In that case, an entry into the population could either be a new entry (new dairy enterprise) or a re-entry as formerly explained. For the purpose of this study, we considered an entry to have occurred when it was preceded by at least two years with missing observations and an exit when it was succeeded by at least two years with missing observations.

2.2. Case definition

A case was defined as one from which *S. agalactiae* was cultured from its BTM sample as previously described. A non-case was that from which the pathogen was not identified by the same bacteriological procedure.

2.3. Statistical analysis

2.3.1. Apparent prevalences

Estimation of the annual apparent prevalences (Ap) and their associated 95% exact binomial confidence intervals was carried out using Stata software (Stata version 11.2, Stata Corporation, College Station TX, USA).

2.3.2. Incidence, entry, exit, recovery and transmission (β) rates

Dohoo et al. (2009) contend that in open populations calculation of rates as opposed to risks is befitting. Consequently, we computed the annual incidence rates based on an approximation of the amount of herd-time at risk for the rate denominator as:

$$Rate = \frac{No.of\ newly\ infected\ herds\ in\ a\ specific\ year\ (cases)}{Average\ no.at\ risk \times time}$$

where:

Average no. at risk = No. at risk at the start of the year + {0.5 × (susceptible entries + recoveries – cases - susceptible exits)}.

The associated 95% confidence intervals (CI) were calculated using an exponential error factor (EF) for incidence rates (Kirkwood and Sterne, 2003):

Lower CI limit: rate × EF^{-1};

Upper CI limit: rate × EF;

where EF = exp(1.96/√d) and *d* is the rate numerator.

Based on the pattern given by the annual incidence rates (Fig. 1), we assessed the significance of the difference between pooled incidence rates before and after 2005 by incorporating a time covariate in both Poisson and negative binomial regression models fitted to the data. The fit of the models were compared using likelihood ratio tests (Dohoo et al., 2009).

However, the rates of entry (σ), exit and recovery (r) were calculated as averages because they were assumed to be constant during the 10-year period (Fig. 2). Both Poisson and negative binomial regression models were used to obtain the average estimates. Notably, a separate exit rate was calculated for non-case (non-infection-related exit rate, μ_j) and case herds (infection-related rate, μ_k). The proportion of entries into the population of non-cases (θ) and cases (1 - θ) was derived by dividing the mean numbers joining the respective populations by the mean number of total entries during the entire period i.e. $n_{non\text{-}cases}/N_E$ and n_{cases}/N_E, where $n_{non\text{-}cases}$ and n_{cases} are the mean number of non-case and case entries respectively and N_E is the mean of the total number of entries.

To estimate the parameter β, we used the framework of an SIS (Susceptible-Infected-Susceptible) model (Fig. 3) for the transmission of *S. agalactiae* between herds. Its use is motivated by the fact that infected herds upon recovery are capable of being re-infected. Thus, the population of dairy herds was accordingly partitioned into S (non-cases) and I (cases) states. New herd infections with *S. agalactiae* were assumed to occur at the rate $\beta \times S \times I/N$, where β is the transmission rate, S the number of susceptible herds, I the number of infected herds and N is the total number of herds present in a specific year (Zadoks et al., 2002). As with the average rates, the number of new infections, C, in each year, was modelled assuming both Poisson and negative binomial distributions:

$\varepsilon[\ln(C)] = \ln[\beta] + \ln[S \times I/N]$

where ε is the expected value and $\ln[S \times I/N]$ was used as a model offset.

Considering the shape of the incidence curve in Fig.1, we also assessed whether the estimate of β differed before and after 2005 by incorporating a time covariate in the models.

3. Results

A frequency distribution of the number of times herds had been infected during the 10-year period is displayed in Table 1. A total of 765 (7.65%) herds had been infected at least once over the course of the study period. The median duration of infection was 2 years.

Descriptive statistics on the annual entry, exit, recovery, incidence and prevalence of *S. agalactiae* are presented in Table 2.

3.1. Apparent prevalences

The annual prevalence estimates of *S. agalactiae* are graphed in Fig. 4. The prevalence increased steadily over time with the lowest and highest values recorded in years 2000 and 2008 respectively.

3.2. Incidence rates

The annual herd-level incidence rates of *S. agalactiae* are displayed in Fig. 1. As with Ap, there was a general increase in the incidence of herd infections over the 10-year period. However, there were two distinctive incidence patterns observed before and after 2005 (P <0.001). As was the case with Ap, the lowest and highest rates were observed in years 2000 and 2008 respectively.

3.3. Entry, exit, recovery and transmission rates.

The herd-level parameter estimates are summarised in Table 3. For all the parameters, a negative binomial regression model provided a better fit to the data than a corresponding Poisson model (P <0.001). The rate at which herds joined the population of dairy herds (0.3 per 100 herd-years [95% confidence interval (CI) 0.2 – 0.4]) was lower than the rate at which they exited (7.1 per 100 herd-years [95% CI 5.6 – 8.9]; 9.2 per 100 herd-years [95% CI 7.4 – 11.5], for infection and non-infection-related rates respectively). A higher proportion of herds joined the susceptible pool of herds (0.97) as compared to the infected one (0.03). The value of the *S. agalactiae*-specific β parameter was estimated to be 54.1 new herd infections per 100 herd-years (95% CI 46.0 – 63.7). This value remained constant before and after 2005 (P = 0.82).

Table 1

Frequency distribution of the number of times herds had been infected during the period 2000 – 2009 in the population of Danish dairy herds.

No. of times infected	0	1	2	3	4	5	6	7	8	9	10
Frequency n (%)	9238 (92.35)	379 (3.79)	142 (1.42)	104 (1.04)	46 (0.46)	35 (0.35)	29 (0.29)	11 (0.11)	4 (0.04)	13 (0.13)	2 (0.02)

Median duration of infection – 2 years.

Table 2

Descriptive statistics on the annual entry, exit, recovery, incidence and prevalence of *Streptococcus agalactiae* in the population of Danish dairy herds.

		[a]Entry		Infection exit & Recovery						Non-infection exit & Incidence						Prevalence	
Year	N	[b]S.ENT	[c]I.ENT	[d]INI	[e]ANI	[f]IE	[g]IEr	[h]REC	[i]RECr	[j]INS	[k]ANS	[l]NIE	[m]NIEr	[n]INC	[o]INCr	[p]INF	[q]Ap
2000	9886	20	0	113	114.0	6	5.3	57	50.0	9947	9559.0	788	8.2	65	0.7	118	1.2
2001	8207	18	0	118	130.0	11	8.5	43	33.1	9768	9140.5	1238	13.5	78	0.9	128	1.6
2002	7662	8	0	128	140.0	11	7.9	38	27.1	8079	7658.5	814	10.6	73	1.0	170	2.2
2003	7329	49	0	170	175.0	12	6.9	65	37.1	7492	7238.5	534	7.4	87	1.2	180	2.5
2004	6627	10	0	180	164.0	13	7.9	75	45.7	7149	6807.5	712	10.5	56	0.8	149	2.3
2005	5416	24	1	149	180.0	20	11.1	46	25.6	6478	5906.0	1087	18.4	127	2.2	207	3.8
2006	5231	10	0	207	197.5	7	3.5	92	46.6	5209	5060.5	319	6.3	80	1.6	195	3.7
2007	4714	21	1	195	196.0	15	7.7	80	40.8	5036	4782.0	513	10.7	96	2.0	196	4.2
2008	4430	13	2	196	204.5	10	4.9	76	37.2	4518	4363.0	298	6.8	101	2.3	213	4.8
2009	4258	20	1	213	205.0	11	5.4	97	47.3	4217	4129.0	202	4.9	91	2.2	198	4.7

[a]Only counts of entries presented as rate denominator was indeterminate; [b]Susceptible entries; [c]Infected entries; [d]Initial no. infected;

[e]Average no. infected; [f]Infection exits; [g]Infection exit rate; [h]Recoveries; [i]Recovery rate; [j]Initial no. susceptible; [k]Average no. susceptible;

[l]Non-infection exits; [m]Non-infection exit rate; [n]Incidence; [o]Incidence rate; [p]No. infected; [q]Apparent prevalence.

IEr; RECr; NIEr; INCr expressed as number of events per 100 herd-years at risk.

Ap expressed as a percentage.

ANI = INI + {0.5 × (I.ENT + INC – REC - IE)}.

ANS = INS + {0.5 × (S.ENT + REC – INC - NIE)}.

Table 3

Parameter estimates for rates of entry, exit, recovery and transmission in an SIS (Susceptible-Infected-Susceptible) model that represents the dynamics of *Streptococcus agalactiae* transmission in the population of Danish dairy herds. Rates of entry, exit, recovery and transmission are expressed as number of events per 100 herd-years at risk.

Parameter	Symbol	Estimate	95% [b]CI	Alpha	Mean	[c]OF
Entry rate	σ	0.3	0.2 – 0.4	0.26	19.8	6.1
Exit rate unrelated to infection	μ_j	9.2	7.4 – 11.5	0.12	650.5	79.1
Exit rate related to infection	μ_k	7.1	5.6 – 8.9	0.05	11.6	1.6
Recovery rate	r	40.0	36.8 – 43.5	0.01	66.9	1.7
Transmission parameter	β	54.1	46.0 – 63.7	0.06	85.4	6.1
[a]Proportion of entries that is susceptible	θ	0.97	-	-	-	-

[a]Fraction; [b]Confidence interval;

[c]Overdispersion factor –calculated as: 1 +(alpha × mean).

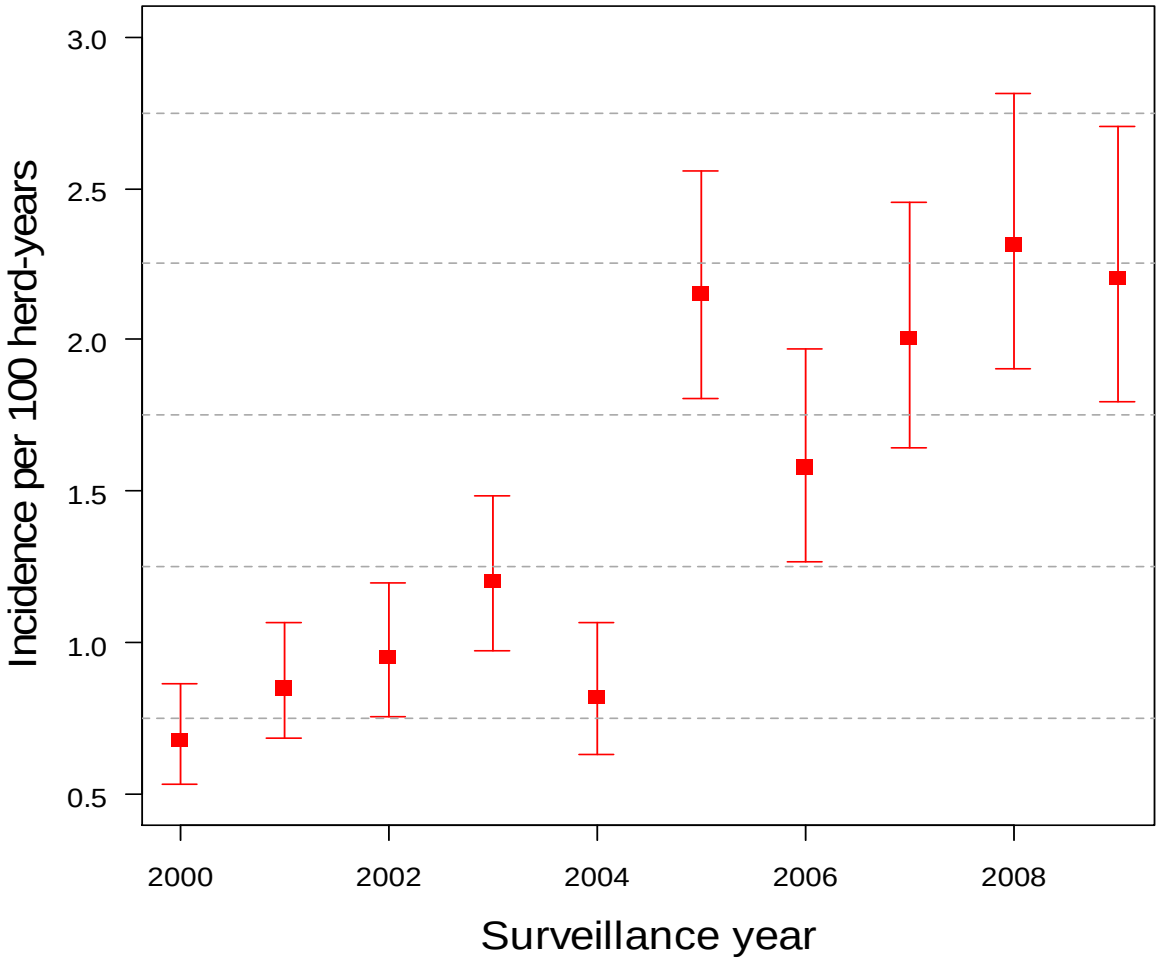

Fig. 1. A plot of the annual herd-level incidence rates of *Streptococcus agalactiae* in the population of Danish dairy herds.

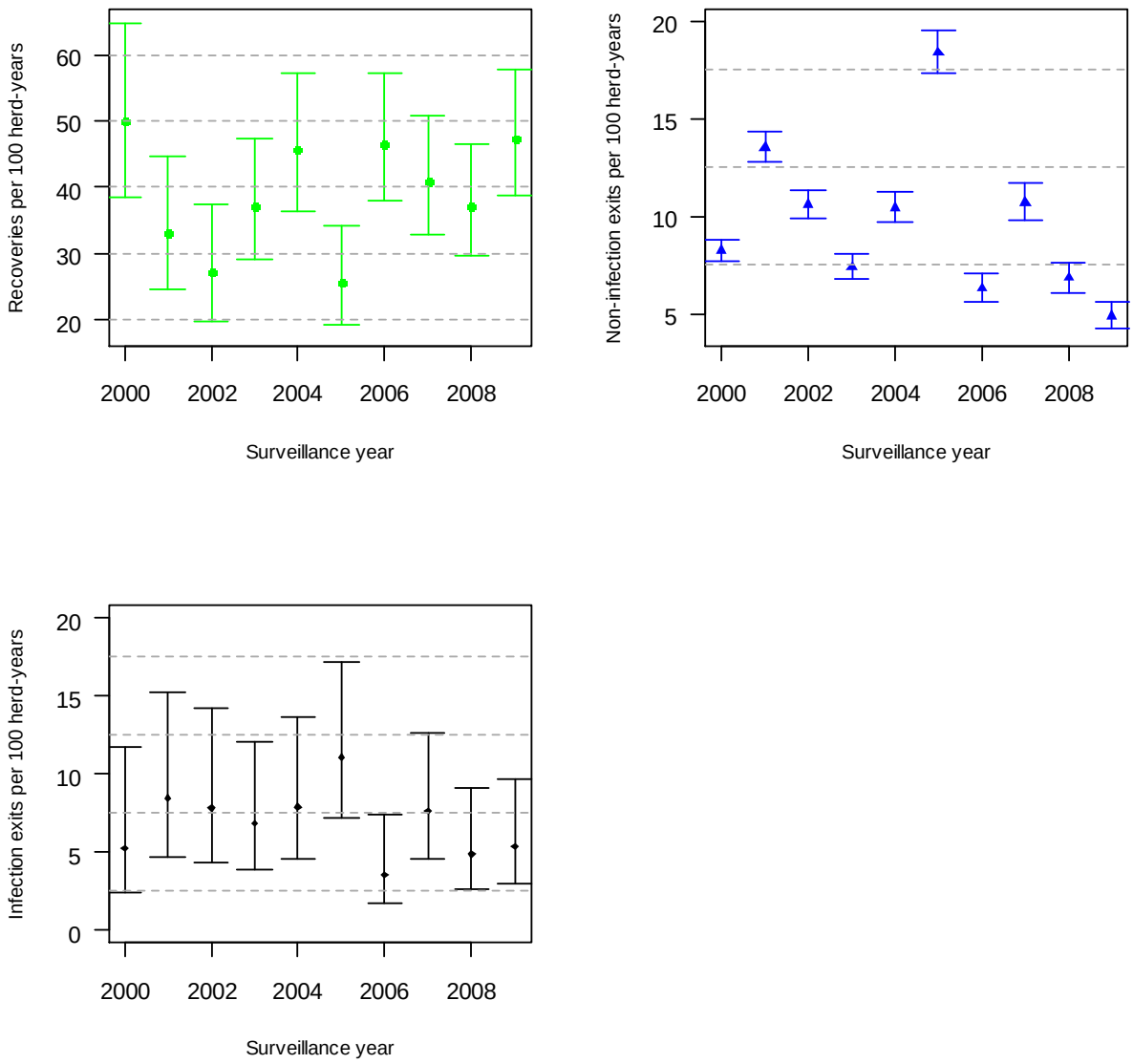

Fig. 2. Plots of the annual herd-level recovery, non-infection and infection exit rates from the population of Danish dairy herds.

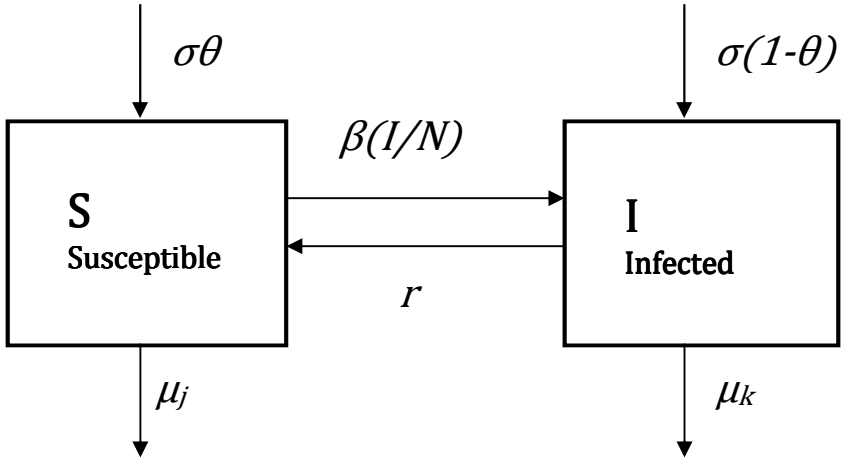

Fig. 3. A schematic representation of the SIS (Susceptible-Infected-Susceptible) model used for the estimation of the transmission parameter, β in the population of Danish dairy herds. The boxes represent the state variables and the arrows represent the flow rates between them. Lettering represents the variables and parameters in the model (Table 3).

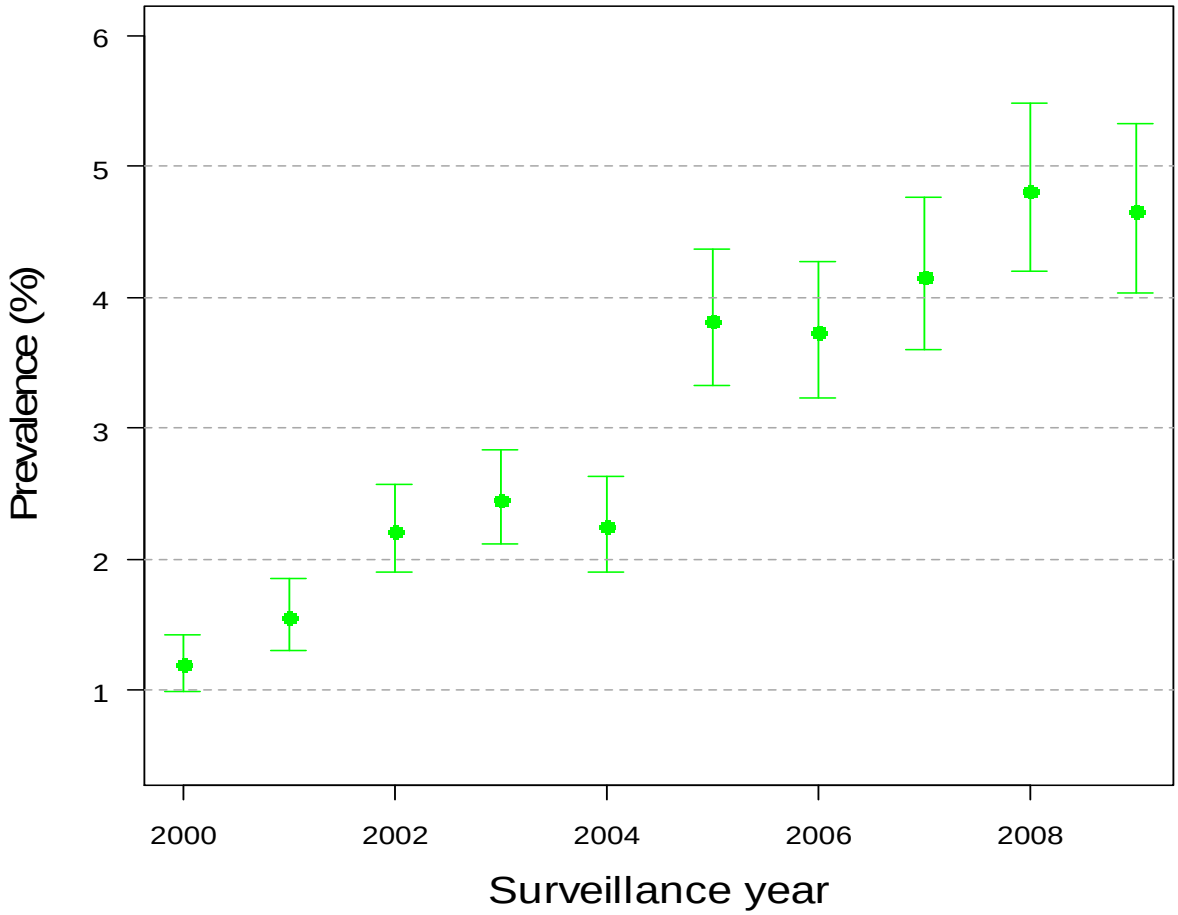

Fig. 4. A plot of the annual herd-level apparent prevalences of *Streptococcus agalactiae* in the population of Danish dairy herds.

4. Discussion

The rising trend in both the incidence and prevalence after a period of quiescence is indicative of a possible re-emergence of the pathogen in the Danish dairy herd population. In Canada and some North-European countries, *S. agalactiae* has been considered to be at the brink of eradication (Pitkälä et al., 2004; Østerås et al., 2006; Piepers et al., 2007; Sampimon et al., 2009; Olde Riekerink et al., 2010). The resurgence of this mastitis problem may be suggestive of either: (1) possible emergence of new and better adapted *S. agalactiae* strains or (2) a breakdown in the stringent implementation of the '5-point plan' for mastitis control developed in the 1960s (Neave et al., 1969). Recent work by Zadoks et al. (2011) involving multi-locus sequence typing of 111 isolates collected from a 2009 Danish BTM survey revealed that the commonest *S. agalactiae* strains were sequence types (ST)1 (28%) and ST23 (23%), which were previously primarily associated with human infection. With respect to adherence to the 5-point mastitis plan, it is particularly noteworthy that only selective dry cow therapy is permitted in Nordic countries (Olsen, 1975; Funke, 1988). In Denmark, the number of dairy herds has been declining with the average herd size steadily increasing. These changing herd dynamics could influence the frequency of *S. agalactiae*. With increasing herd size, the demand for labour is expected to rise. This may bring about a heightened risk of infection for herds as humans may serve as a source of infection for cattle (Zadoks and Schukken, 2006). This situation is expected if on-farm biosecurity measures are not commensurately beefed up. Moreover, a rapid herd expansion may increase the need for purchasing animals which may result in elevated risk of introducing *S. agalactiae*. There were 2 constant incidence patterns observed in the dairy herd population with the transition between them marked by a sudden surge in 2005. This observation coincides with a Danish ministerial directive issued in the same year (Anonymous, 2005), which authorised the lifting of a movement ban imposed on *S. agalactiae* infected herds. However, according to the order, farmers with infected herds were thereafter obligated to disclose their herd status to all coming into contact with their respective herds. Indeed, in light of our results, there is a pressing need to review the current policy pertaining to the control of *S. agalactiae*.

The parameter estimates in this study correspond to an R_o value of 1.1 ($\beta/r + \mu_k$). This implies that on average each infected herd leads to more than one new herd infection in a susceptible population of Danish dairy herds. Therefore, given the value of R_o it would be generally expected that incidence would increase as was exemplified by the shape of the incidence curve in Fig. 1. Successful control of transmission is said to be achieved when R_o is reduced to a value below unity, which signifies that infection would not persist. This study thus illustrates a need to strengthen existing control measures against *S. agalactiae* in order to reduce R_o, and hence incidence. However, it remains debatable as to whether elimination of *S. agalactiae* in this population is feasible even in the absence of infected animal movements since infection from humans is still a threat.

In the estimation of β, we implicitly assumed homogeneous mixing of herds. This assumption could have been violated in two ways: (1) the data were overdispersed suggesting the possibility of clustering and (2) the fact that farms are spatially distributed entities, in which case assuming such a mixing pattern would be erroneous (Heath et al., 2008). However, Newman (2002) shows that in a network setting, the β parameter can be estimated in an analogous way only that its interpretation would be slightly different; the rate of transmission from an infective node (herd) to a susceptible node along a given edge (e.g. infected movement) per unit of time. Thus, the R_o estimated from a non-network setting would have to be adjusted for the average number of

neighbours each farm trades with minus 1. The minus 1 accounting for the fact that every infectious herd, except for the initial infective, must have acquired infection from one of its neighbours (Diekmann et al., 1998). Additionally, the computed β is an aggregate parameter encompassing all potential sources of transmission. Although calculation of source-specific transmission parameters would have been preferable, this was not possible in our study as only aggregated data on the number of newly infected herds were available. Thus from the estimated β, it is difficult to deduce the relative importance of the individual sources in the transmission mechanism of the pathogen.

Bacteriological culture does not correctly identify all infected herds because its sensitivity is low (Mweu et al., 2012). We attempted a manual adjustment for misclassification in the parameters of the present study by changing the status of herds that had positive-negative-positive profiles to positive-positive-positive. This adjustment affected incidence, exit and recovery rates (data not shown). For instance, the incidence rate in 2009 decreased from 2.2 cases per 100-herd years to 1.9 per 100 herd-years whereas the infection-related exit and recovery rates reduced to 6.5 and 30.2 per 100 herd-years respectively. This adjustment method cannot be validated, and there is presently no other available means for adjustment. Nevertheless, the results provide a sneak insight into the magnitude of misclassification inherent in the estimates. Thus, the study findings are "apparent" and can only be strictly treated as such.

5. Conclusion

There was an increasing trend in both the incidence and prevalence of *S. agalactiae* in the Danish dairy herd population between 2000 and 2009. We also estimated parameters that can be used to model the effectiveness of control strategies against *S. agalactiae* transmission.

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CHAPTER 3b:

DIAGNOSTIC TEST-ADJUSTED ANNUAL INCIDENCE AND PREVALENCE OF *STREPTOCOCCUS AGALACTIAE* INFECTION IN DANISH DAIRY HERDS: 1966 – 2011

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Preamble

This chapter is a follow-up to chapter 3a that aims to adjust the annual herd-level apparent estimates of incidence and prevalence of *Streptococcus agalactiae* (*S. agalactiae*) for diagnostic misclassification. The analysis is also extended to cover 1966 – 1999 and 2010 – 2011 periods.

Abstract

Estimates of incidence and prevalence of infections obtained from population-based surveys using imperfect diagnostic tests are bound to be impacted by misclassification errors. Failure to acknowledge and account for these test errors may lead to under or overestimation of infection frequencies and thus inappropriate allocation of resources in surveillance and control programmes for the infections. Therefore, the objective of this study was to estimate the annual herd-level true incidence risks and prevalences of *S. agalactiae* infection in the population of Danish dairy herds during the period from 1966 – 2011 inclusive.

A Bayesian framework was employed to analyse BTM surveillance data on *S. agalactiae* spanning the period 1966 – 2011 inclusive. The results showed that, during the culture-based phase of the surveillance programme between 1966 and 2008, the apparent prevalences (Ap) consistently underestimated the true prevalences (Tp), while conversely the apparent incidence risks (A_i) overestimated the true incidence risks (T_i) of the infection. During the PCR phase of the scheme between 2009 and 2011, the Aps however overestimated the Tps whereas the A_is remained as overestimates of the T_is of the infection. Of note, fluctuations in the Tp and T_i trends closely paralleled legislative changes relating to *S. agalactiae* control. There were decreasing trends in the Tp and T_i of the infection subsequent to the introduction of PCR surveillance of BTM samples in 2009. Bacteriological culture showed suitability for use in identifying new herd infections but demonstrated insufficiency for detecting existing infections. Contrastingly, the PCR assay showed adequacy for detecting both new and existing herd infections and thus offers better promise for facilitating control efforts aimed at eliminating *S. agalactiae* infections from Danish dairy herds.

Keywords: Bayesian analysis; Cattle; *Streptococcus agalactiae*; Test misclassification; True incidence; True prevalence

1. Introduction

Efforts to establish the frequency of infections in population-based surveys using diagnostic tests often result in apparent (test) estimates of prevalence (Ap) and incidence (A_i) that are plagued by misclassification errors since screening tests are seldom perfect (Speybroeck et al., 2013). Failure to acknowledge and account for these test errors (false positives and negatives) can lead to imprecise sample size calculations, biased estimates of measures of effects in risk-factor studies, under or overestimation of infection frequencies and hence inappropriate allocation of resources in surveillance and control programmes (McV Messam et al., 2008). With availability of information on the accuracy of the tests used, corrections of the Ap and A_i to the true prevalence (Tp) and incidence risk (T_i) of infections respectively, can be made.

Frequentist and Bayesian approaches are available for the derivation of the Tp based on the Ap, test sensitivity (Se) and specificity (Sp) (Crowling et al., 1999). The Rogan-Gladen estimator (Rogan and Gladen, 1978) – the classical frequentist method – has long been upheld for its simplicity although it suffers two important drawbacks: (1) it requires that values of Se and Sp of the test are known and fixed, which is impractical in most cases and (2) implausible negative Tp values may occur in situations where the Ap is lower than the false positive fraction (1 – Sp) (Hilden, 1979). By contrast, in a Bayesian framework, the test Se and Sp are modelled as probability distributions and as such their uncertainties are incorporated. Moreover, by combining the observed data on Ap with a priori information on the Se and Sp, probability rule-respecting posterior estimates can be inferred (Crowling et al., 1999). Owing to these merits, the appeal for Bayesian inference in prevalence estimation is increasingly growing (Staubach et al., 2002; Dorny et al., 2004; Dhand et al., 2010; Nielsen et al., 2011).

In the estimation of incidence in longitudinal studies, it is common practice to exclude subjects that are test positive for infection at baseline from subsequent follow-up. The goal is often to ensure that individuals testing positive at follow-up (deemed 'infected') have initially been 'free' of the infection. However, the use of imperfect diagnostic tests is bound to introduce misclassification errors both at baseline (where false negative and false positive subjects are erroneously included and omitted respectively, from follow-up) and follow-up, biasing computed estimates of incidence. In addition, as false negative individuals recruited at baseline may recover from infection during follow-up, adjustments to the Ti of infection should factor in the sensitivities and specificities of the tests used at baseline and follow-up, baseline Tp of infection and the true probability of recovery from infection during follow-up (Pekkanen et al., 2006). As with Tp estimation, a Bayesian approach for Ti derivation can be adopted to circumvent the aforementioned frequentist demerits.

Since the inception of the Danish *S. agalactiae* surveillance programme, bacteriological culture has served as the standard screening tool of bulk tank milk (BTM) samples. However, recently the programme has incorporated the use of a novel, rapid PCR assay – the PathoProof Mastitis PCR assay – which has been considered to hold better promise for use in routine *S. agalactiae* screening given its comparably high Se (Mweu et al., 2012a). Even though trends in the herd-level incidence and prevalence of *S. agalactiae* have been estimated (Mweu et al., 2012b), the estimates were nonetheless unadjusted for test misclassification. Therefore, the objective of this study was to estimate the annual herd-level true incidence risks and

prevalences of *S. agalactiae* infection in the population of Danish dairy herds during the period from 1966 – 2011 inclusive.

2. Materials and methods

2.1 Data

BTM surveillance data on *S. agalactiae* spanning the period 1966 – 2011 inclusive, were extracted from three key sources: (1) the Veterinary Directorate annual reports for the period 1966 – 1982 (Anon., 1966-1982), (2) annual reports on mastitis control for the period 1983 – 1990 (Anon., 1983-1990) and (3) the Danish Cattle Database (DCD) for data covering the period 1991 – 2011. Of note, data from the period 1966 to 1982 were collected based on a voluntary surveillance scheme that began with an initial enrolment of 30.9% of the Danish dairy herd population in 1966 and gradually grew over time to peak at 94.5% in 1982 (Anon., 1966-1982). Data for the subsequent period i.e. 1983 – 2011 were collected as part of the mandatory surveillance programme for *S. agalactiae* and hence comprised all the registered dairy herds. For the annual reports, along with the number and proportion of participating herds, the proportion of the herds infected as well as cleared of the infection were recorded. Importantly, the data from 1966 to 2008 were based on BTM bacteriological culture results whereas those from 2009 to 2011 were PCR based.

As pertains to the DCD data, granted that repeat testing was effected for some herds in certain years, to guarantee consistency with the rest of the data, the first test result for a particular herd in a given year formed the basis for classifying herds. By definition, for years in which herds were screened by culture, a case was a herd from which *S. agalactiae* was isolated from its BTM sample; a non-case being contrarily defined. As for the PCR data, since at a Ct cut-off value of 40 the assay has been shown to have the highest combined accuracy (Mweu et al., 2012a), herds whose outcomes fell below this threshold were deemed positive for *S. agalactiae*. To obtain an incidence dataset, cases were regarded as new in a given follow-up year only if they had been non-cases in the baseline year. It should be noted that only the DCD data could facilitate computation of incidence risks because information on individual herds was available.

2.2 Statistical analysis

In the estimation of incidence, the annual Tis were derived from the corresponding annual A_is by adjusting for the test misclassification both at the baseline and follow-up stages, while accounting for the recovery of false negatives during follow-up (Pekkanen et al., 2006). Similarly, in prevalence estimation, the annual Aps were corrected for the sensitivities and specificities of the tests to obtain the annual Tps. The derivations were achieved using a Bayesian model fitted in OpenBUGS software (Thomas et al., 2006) as follows:

$$\mathrm{Inc}_{t+1} \sim \mathrm{Binomial}(\mathrm{A}_{\mathrm{i}t+1}, \mathrm{POPr}_t)$$

$$\mathrm{A}_{\mathrm{i}t+1} = \frac{\mathrm{Se}_2[\mathrm{Sp}_1(1-\mathrm{Tp}_t)\mathrm{T}_{\mathrm{i}_{t+1}} + (1-\mathrm{Se}_1)\mathrm{Tp}_t(1-r_{t+1})] + (1-\mathrm{Sp}_2)[\mathrm{Sp}_1(1-\mathrm{Tp}_t)(1-\mathrm{T}_{\mathrm{i}_{t+1}}) + (1-\mathrm{Se}_1)\mathrm{Tp}_t r_{t+1}]}{\mathrm{Sp}_1(1-\mathrm{Tp}_t) + (1-\mathrm{Se}_1)\mathrm{Tp}_t}$$

$$\mathrm{Y}_t \sim \mathrm{Binomial}(\mathrm{Ap}_t, \mathrm{N}_t)$$

$$\mathrm{Ap}_t = \mathrm{Se}_1\mathrm{Tp}_t + (1 - \mathrm{Sp}_1)(1 - \mathrm{Tp}_t)$$

With prior information:

$Se_{CUL} \sim Beta(a_{CULse}, b_{CULse})$

$Se_{PCR} \sim Beta(a_{PCRse}, b_{PCRse})$

$Sp_{CUL} \sim Beta(a_{CULsp}, b_{CULsp})$

$Sp_{PCR} \sim Beta(a_{PCRsp}, b_{PCRsp})$

$T_{i(t+1)} \sim Beta(1, 1)$

$r_{t+1} \sim Beta(1, 1)$

$Tp_t \sim Beta(1, 1)$

where Inc_{t+1} and Y_t denote the number of observed new and existing cases in years t+1 (follow-up year) and t (baseline year), $A_{i(t+1)}$ and Ap_t are the apparent incidence risks and prevalences in years t+1 and t, $POPr_t$ and N_t refer to the sizes of the population at risk and the total population in year t, Se_1, Se_2, Sp_1 and Sp_2 are sensitivities and specificities of the tests used at baseline and follow-up, $T_{i(t+1)}$ and Tp_t are the true incidence risks and prevalences in years t+1 and t and r_{t+1} is the true probability of infection recovery during follow-up in year t+1. In particular, depending on the pair of years under consideration, bacteriological culture and/or PCR served as the baseline and follow-up screening tests. The prior distributions of the Se and Sp of culture and PCR (Se_{CUL}, Sp_{CUL} and Se_{PCR}, Sp_{PCR}) were modelled as beta distributions. Values of the alpha ($a_{CUL/PCR}$) and beta ($b_{CUL/PCR}$) parameters were elicited using the BetaBuster software available at: http://www.epi.ucdavis.edu/diagnostictests/betabuster.html, based on estimates of Se and Sp of culture and PCR reported in Mweu et al. (2012a) (Table 1). Beta(1, 1) distributions were applied for the priors of T_i, r and Tp which are essentially uniform on a 0 – 1 interval, to reflect an absence of their prior knowledge.

Two Markov Chain Monte Carlo (MCMC) chains initialised with different values were used to fit the model. Each chain comprised 120,000 samples, of which the first 20,000 were discarded as the burn-in. Convergence of the MCMC chains was assessed by visual appraisal of the time-series plots of selected variables and the Gelman-Rubin diagnostic plots.

Table 1

The mode and 5th percentile values of the sensitivity (Se) and specificity (Sp) of bacteriological culture (Se/Sp_{CUL}) and PCR (Se/Sp_{PCR}) as well as their corresponding alpha and beta parameter values for the Beta probability distributions generated using the BetaBuster software.

Test	Mode	5th percentile	Alpha parameter	Beta parameter
Se_{CUL}	0.680	0.551	29.05	14.20
Sp_{CUL}	0.997	0.993	1258.87	4.78
Se_{PCR}	0.952	0.882	60.01	3.98
Sp_{PCR}	0.988	0.972	306.17	4.71

3. Results

Within a span of 45 years, the Danish dairy herd population declined drastically from over 136,000 in 1966 to just under 4,000 in 2011 (Table 2). Between 1966 and 2008, the Ap consistently underestimated the Tp, while by contrast the A_i overestimated the T_i of the *S. agalactiae* infection. Nevertheless, between 2009 and 2011, the Ap overestimated the Tp, while the A_i remained an overestimate of the T_i of the infection (Table 2).

Between 1966 and 1981, a declining trend in the Tp was observable from slightly above 4% in 1966 to 2% in 1981 (Fig. 1). In 1982/83, there was an initial increase in the Tp followed by a decline to 1% in 1989. Afterwards, the Tp rose to peak at 2.1% in 1992. This was succeeded by a drop in Tp to a level of 1% in 1995, with the trend remaining relatively stable below 1.4% until 1999. As from 2000, the Tp increased dramatically to peak at over 6% in 2009. In particular, during this period, a characteristic surge in Tp was noticeable in 2005. Afterwards, a decreasing trend in Tp was observable. As from 1993 (except for 2001), the trend in the T_i remained at a fairly stable level at/below 0.5% until 2004. Subsequently, the T_i rose to above 2% in 2005, but then, with the exception of 2006, dropped to stay above the 0.9% mark (Fig. 1).

Table 2

Estimated annual herd-level true prevalences and incidence risks of *S. agalactiae* in the population of Danish dairy herds during the period 1966 - 2011.

Year	[a]N (*)	[b]INF	[c]AP (95% [d]PCI)	[e]TP (95% PCI)	[f]POPr	[g]INC‡	[h]AI (95% PCI)	[i]TI (95% PCI)
1966	136731 (42250)	1352	3.20 (3.04 - 3.37)	4.12 (3.67 - 4.52)	-	-	-	-
1967	121124 (43968)	1363	3.10 (2.94 - 3.27)	3.98 (3.54 - 4.38)	-	-	-	-
1968	116380 (51207)	1485	2.90 (2.76 - 3.05)	3.71 (3.29 - 4.08)	-	-	-	-
1969	105944 (54667)	1476	2.70 (2.57 - 2.84)	3.44 (3.04 - 3.79)	-	-	-	-
1970	95782 (52680)	1317	2.50 (2.37 - 2.64)	3.17 (2.79 - 3.51)	-	-	-	-
1971	75867 (52500)	1365	2.60 (2.47 - 2.74)	3.30 (2.91 - 3.65)	-	-	-	-
1972	71571 (56040)	1401	2.50 (2.37 - 2.63)	3.17 (2.79 - 3.50)	-	-	-	-
1973	64498 (50760)	1269	2.50 (2.37 - 2.64)	3.17 (2.79 - 3.51)	-	-	-	-
1974	60581 (49192)	1279	2.60 (2.46 - 2.74)	3.30 (2.91 - 3.65)	-	-	-	-
1975	55852 (48200)	1205	2.50 (2.37 - 2.64)	3.17 (2.79 - 3.51)	-	-	-	-
1976	54207 (46130)	1061	2.30 (2.17 - 2.44)	2.90 (2.53 - 3.23)	-	-	-	-
1977	48614 (41857)	879	2.10 (1.97 - 2.24)	2.63 (2.27 - 2.95)	-	-	-	-
1979	39351 (34826)	801	2.30 (2.15 - 2.46)	2.90 (2.51 - 3.25)	-	-	-	-
1980	36373 (31895)	606	1.90 (1.76 - 2.06)	2.35 (2.00 - 2.68)	-	-	-	-
1981	35121 (32875)	526	1.60 (1.47 - 1.74)	1.95 (1.62 - 2.24)	-	-	-	-
1982	34235 (33643)	942	2.80 (2.63 - 2.98)	3.57 (3.15 - 3.97)	-	-	-	-
1983	33111	894	2.70 (2.53 - 2.88)	3.44 (3.02 - 3.83)	-	-	-	-
1984	31250	625	2.00 (1.85 - 2.16)	2.49 (2.13 - 2.82)	-	-	-	-
1985	28267	424	1.50 (1.37 - 1.65)	1.81 (1.49 - 2.11)	-	-	-	-
1986	26467	397	1.50 (1.36 - 1.65)	1.81 (1.49 - 2.11)	-	-	-	-
1987	24833	298	1.20 (1.07 - 1.34)	1.41 (1.10 - 1.69)	-	-	-	-
1988	22600	226	1.00 (0.88 - 1.14)	1.14 (0.85 - 1.40)	-	-	-	-
1989	22444	202	0.90 (0.78 - 1.03)	1.00 (0.72 - 1.26)	-	-	-	-
1990	20091	221	1.10 (0.97 - 1.25)	1.27 (0.97 - 1.56)	-	-	-	-
1991	19168	292	1.53 (1.36 - 1.71)	1.85 (1.50 - 2.18)	-	-	-	-
1992	17570	302	1.72 (1.54 - 1.92)	2.11 (1.74 - 2.46)	16963	182	1.12 (0.97 - 1.28)	0.63 (0.37 - 0.90)
1993	16127	241	1.50 (1.32 - 1.69)	1.81 (1.45 - 2.15)	15792	142	0.91 (0.78 - 1.06)	0.25 (0.03 - 0.52)
1994	15850	204	1.28 (1.12 - 1.45)	1.51 (1.18 - 1.82)	15129	139	0.92 (0.78 - 1.09)	0.38 (0.12 - 0.65)
1995	14370	131	0.92 (0.77 - 1.08)	1.02 (0.72 - 1.31)	14157	91	0.66 (0.56 - 0.80)	0.13 (0.01 - 0.35)
1996	13419	147	1.08 (0.92 - 1.26)	1.24 (0.92 - 1.55)	13262	97	0.74 (0.60 - 0.89)	0.41 (0.18 - 0.65)
1997	12141	112	0.93 (0.77 - 1.11)	1.04 (0.72 - 1.34)	11903	65	0.58 (0.48 - 0.71)	0.11 (0.01 - 0.32)
1998	11527	131	1.14 (0.96 - 1.34)	1.32 (0.98 - 1.66)	11372	76	0.67 (0.54 - 0.84)	0.32 (0.09 - 0.57)
1999	10060	113	1.13 (0.94 - 1.34)	1.31 (0.95 - 1.66)	9884	69	0.71 (0.57 - 0.88)	0.26 (0.04 - 0.54)
2000	9886	118	1.20 (1.00 - 1.43)	1.40 (1.04 - 1.78)	9138	65	0.72 (0.57 - 0.91)	0.29 (0.05 - 0.57)
2001	8207	128	1.57 (1.31 - 1.85)	1.90 (1.47 - 2.35)	8076	78	0.97 (0.78 - 1.20)	0.60 (0.28 - 0.94)
2002	7662	170	2.23 (1.91 - 2.57)	2.79 (2.27 - 3.32)	7068	73	1.04 (0.83 - 1.30)	0.51 (0.16 - 0.89)
2003	7329	180	2.35 (2.04 - 2.69)	2.96 (2.47 - 3.45)	6924	87	1.27 (1.03 - 1.55)	0.49 (0.09 - 0.94)
2004	6627	149	2.26 (1.92 - 2.64)	2.83 (2.29 - 3.42)	6416	56	1.03 (0.85 - 1.24)	0.09 (0.00 - 0.37)
2005	5416	207	3.77 (3.31 - 4.27)	4.89 (4.17 - 5.60)	5246	127	2.43 (2.04 - 2.87)	2.06 (1.47 - 2.69)
2006	5231	195	3.74 (3.25 - 4.27)	4.83 (4.06 - 5.66)	4873	80	1.73 (1.43 - 2.08)	0.30 (0.02 - 0.90)
2007	4714	196	4.17 (3.62 - 4.76)	5.42 (4.56 - 6.33)	4495	96	2.15 (1.76 - 2.60)	0.94 (0.24 - 1.66)
2008	4430	213	4.83 (4.23 - 5.51)	6.30 (5.41 - 7.33)	4201	101	2.42 (1.99 - 2.91)	1.08 (0.30 - 1.88)
2009	4258	310	[j]7.26 (6.57 – 8.03)	6.52 (5.07 - 7.87)	4015	185	[j]4.63 (4.01 – 5.30)	1.79 (0.38 - 3.10)
2010	4091	270	[j]6.60 (5.90 - 7.34)	5.80 (4.38 - 7.04)	3769	129	[j]3.44 (2.89 - 4.06)	1.76 (0.49 - 2.89)
2011	3918	243	[j]6.22 (5.49 - 7.00)	5.36 (3.93 - 6.70)	3633	105	[j]2.92 (2.41 - 3.49)	1.24 (0.14 - 2.35)

[a]Total dairy herd population; *No. tested; [b]No. infected; [c]Apparent prevalence; [d]Posterior credibility interval; [e]True prevalence; [f]Population at risk; [g]Incidence; [h]Apparent incidence risk; [i]True incidence risk; [j]Based on PCR.

AP; TP; AI; TI are median estimates and are expressed as percentages.

‡Incidence data only available as from 1992.

Data for 1978 were missing.

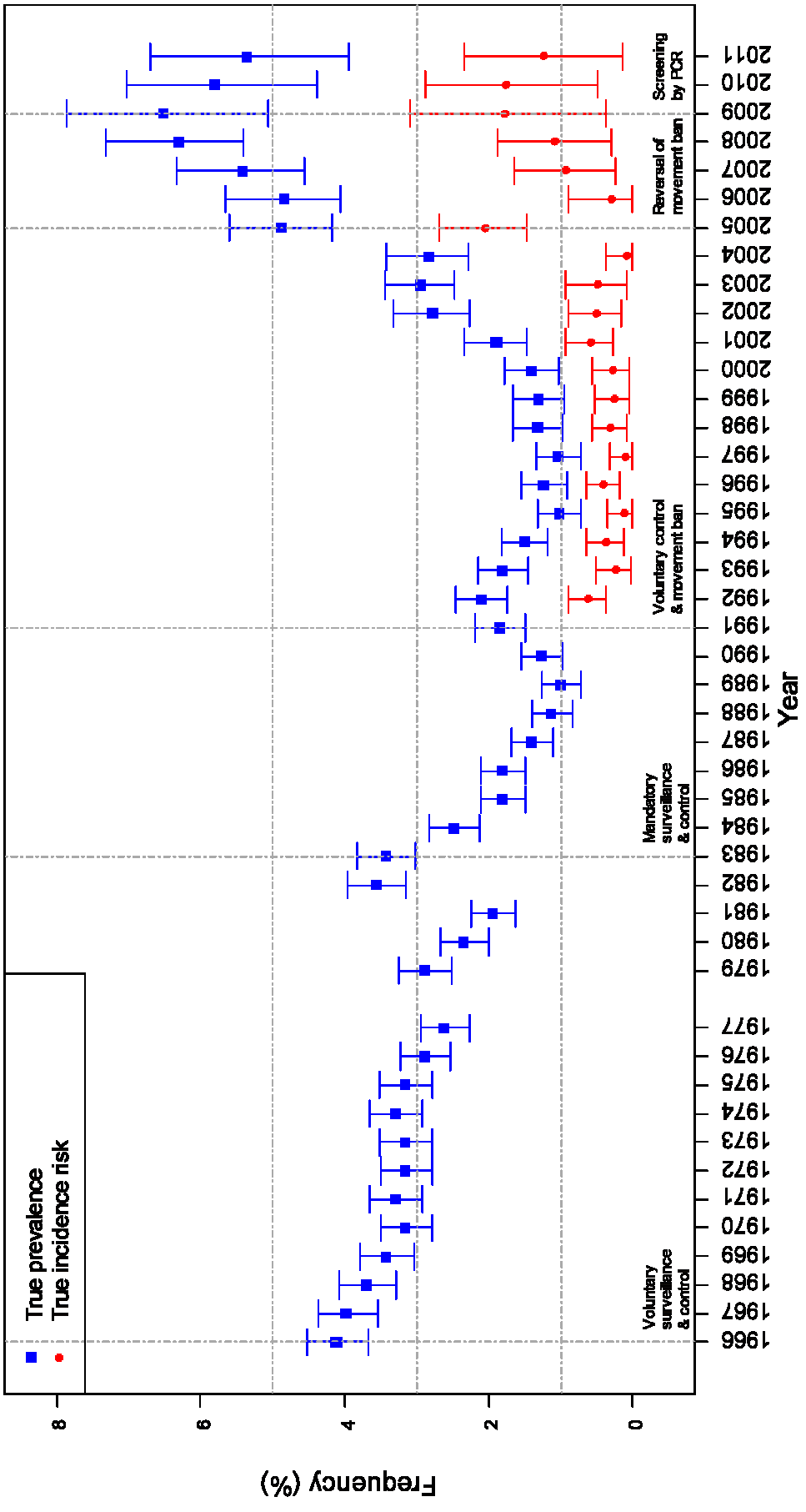

Fig. 1. Plot of the estimated annual herd-level true prevalences and incidence risks of *S. agalactiae* in the population of Danish dairy herds.

4. Discussion

By employing a Bayesian framework, the annual herd-level T_is and Tps have been estimated by correcting their associated apparent estimates for diagnostic test errors. During the period 1966 – 2008, the Ap was shown to consistently underestimate the Tp, whereas the A_i overestimated the T_i of the *S. agalactiae* infection. This period corresponded to the culture-based phase of the surveillance programme. The result that the Ap was lower than the Tp highlights the inadequacy of single bacteriological screens of the BTM in detection of existing infections in the Danish dairy herd population. As a consequence, repeated BTM cultures combined with parallel interpretation of the test results should serve to enhance the culture Se (Godkin and Leslie, 1993; Keefe et al., 1997). Since the primary goal of the *S. agalactiae* surveillance scheme is to improve detection of infected herds for control i.e. minimise the false negative fraction, the finding that A_i was higher than T_i, suggests that single cultures of the BTM could however be well suited for identifying new herd infections. This view appears to be at odds with that of Andersen et al. (2003) who underscored the need for longitudinal examinations of bulk tanks in enhancing the detection of new infections in Danish dairy herds. This contradictory perspective may in part be attributable to the fact that their study did not estimate T_is that could facilitate comparisons with the apparent estimates. Nonetheless, to reduce the proportion of falsely identified new infections, confirmation of positively-testing herds is necessary.

Between 2009 and 2011, a period during which testing of BTM samples was based on PCR, Ap was found to overestimate the Tp, while the A_i remained an overestimate of the T_i. This suggests that, unlike culture, the PCR assay is suited for detecting both existing and new infections in the Danish dairy herd population for which control actions should be taken. Furthermore, the PCR assay affords better promise than bacteriological culture given that, in both cases, single BTM PCR tests appear satisfactory – a switch that could facilitate the elimination of *S. agalactiae* infections from the Danish dairy herd population. Notwithstanding, in order to reduce the proportion of false positives, there is need to confirm PCR-positive herds by culture.

The differences in the tests' detection abilities of existing infections could be because infected cows in such herds have had sufficient time to mount immune responses capable of reducing the amounts of bacteria shed into the BTM to levels which undermine their detection by culture, but could still be sufficiently high for detection by PCR. Conversely, cows in newly infected herds are likely to be shedding fairly high amounts of bacteria detectable by both tests.

The declining trend in the Tp between 1966 and 1981 coincided with the inception of a voluntary surveillance and control programme for *S. agalactiae* in Denmark (Anon., 1966-1982). Campaigns to boost herd enrolment levels together with ensuring increased involvement in active control of infections amongst participating herds could have been responsible for the decline. The initial rise in Tp in 1983 corresponded to the coming into effect of the mandatory surveillance and control for the pathogen (Anon., 1983), which saw all the registered dairy herds in the country enrolled. Afterwards, there was a precipitous drop in Tp attributable to the programme's rigorous efforts in containing infections. The reversion to a voluntary control scheme in 1991 (Anon., 1991) was firstly characterised by an increase in Tp in 1992. But probably owing to a concurrent introduction of a movement ban (an incentive compelling infected herds to maintain control efforts), the Tp declined and remained at a stable level below 1.4% until 1999. Between 2000 and 2009, a dramatic increase in

Tp was observed, with a sudden surge being notable in 2005. The distinguishable increase in 2005 coincides with a reversal of the movement ban whose lifting was considered because it was reasoned that the risk of transmission via purchase of animals was negligible (Anon., 2005a, b). This change could have led farmers to downplay the infection and therefore loosen their grip on the control of *S. agalactiae* resulting in the increase in Tp. However, afterwards, decreasing trends in Tp as well as T_i were witnessed. The decreases could be ascribed to a range of factors: (1) incorporation of PCR in BTM surveillance that could have enhanced detection of existing and new infections for control, (2) intensification of awareness campaigns concerning *S. agalactiae* control amongst farmers and veterinarians in 2009 and 2010 and (3) shortening of sample processing times at the laboratory as from 2010, which could have minimised the risk of laboratory contamination of samples and hence the number of false positives (Katholm and Bennedsgaard, 2011).

Admittedly, this study is not without limitations. The use of data retrieved from reports to infer the annual prevalences between 1966 and 1990 precluded an independent scrutiny of the raw data and as such the accuracy of the computed estimates may be questionable. Moreover, as the voluntary phase of the surveillance scheme (1966 – 1982) only featured a sample of dairy farms and considering that the more problematic herds would be less inclined to participate, the prevalence of the infection is likely to be higher than was observed. An implicit assumption in estimating the T_i and Tp is that the BTM culture classification protocols remained unchanged over time and hence the test's Se and Sp. Godkin and Leslie (1993) remark that the accuracy of culture may vary due to differences in classification of negative and positive culture outcomes. Granted the likely temporal variations in culture-based case definitions, the true estimates may be biased.

5. Conclusion

True estimates of incidence and prevalence of *S. agalactiae* infections in the Danish dairy herd population have been inferred from a Bayesian analysis of longitudinal data extending over the period 1966 – 2011 inclusive. During the culture-based phase of the surveillance programme (1966-2008), the Aps were shown to consistently underestimate the Tps, while conversely the A_is overestimated the T_is of the *S. agalactiae* infection. During the PCR phase of the scheme between 2009 and 2011, the Aps however overestimated the Tps whereas the A_is remained as overestimates of the T_is of the infection. Notably, fluctuations in the Tp and T_i trends closely followed legislative changes pertaining to *S. agalactiae* control. There were decreasing trends in the Tp and T_i of the infection subsequent to the introduction of PCR surveillance of BTM samples in 2009. Bacteriological culture demonstrated capability for identifying new herd infections but was insufficient for detecting existing infections. Contrastingly, the PCR assay showed suitability for detecting both new and existing herd infections and thus offers better promise for facilitating control efforts aimed at eliminating *S. agalactiae* infections from Danish dairy herds.

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CHAPTER 4:

TEMPORAL CHARACTERISATION OF THE NETWORK OF DANISH CATTLE MOVEMENTS AND ITS IMPLICATION FOR DISEASE CONTROL: 2000 – 2009

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Abstract

Social network analysis provides a valuable framework for understanding the dynamics of diseases on networks as well as a means for defining effective control measures. An understanding of the underlying contact pattern for a susceptible population is advisable before embarking on any strategy for disease control. The objective of this study was to characterise the network of Danish cattle movements over a 10-year period from 2000 to 2009 with a view to understanding: (1) cohesiveness of the network, (2) influential holdings and (3) structural vulnerability of the network.

Network analyses of data involving all cattle movements in Denmark registered during the period of interest were performed. A total of 50,494 premises participated in 4,204,895 individual movements during the 10-year period. The results pointed to a predominantly scale-free structure of the network; though marked by small-world properties in March to April 2001 as well as in 24 other months during the period October 2006 to December 2009. The network was sparsely connected with markets being the key influential holdings. Its vulnerability to removal of markets suggests that targeting highly connected holdings during epidemics should be the focus of control efforts.

Keywords: Network analysis; Temporal characteristics; Scale-free; Small-world; Cattle movements.

1. Introduction

A crucial step in stemming the spread of contagious pathogens between animal holdings involves a combined process of singling out and application of rigorous control measures on potentially exposed and infected premises. Movement of animals between holdings constitutes an efficient route, by which many of these pathogens are disseminated. Consequently, having a system in place for the identification and registration of both holdings and animals is a key prerequisite for surveillance and control programmes for contagious diseases (Ammendrup and Barcos, 2006). Availability of such detailed registry data on individual animal displacements (as is found in national livestock databases of European Union (EU) member states) allows a thorough characterisation of their behaviour in time, which may reveal patterns that are relevant for the spread and control of diseases in populations (Natale et al., 2009). Such was the case in the United Kingdom (UK) following the 2001 foot-and-mouth disease (FMD) epidemic where post-hoc analyses showed that market closure coupled with a movement ban on all livestock were more effective containment strategies than a reactive culling policy owing to the prevailing heterogeneous contact structure in the population (Shirley and Rushton, 2005).

Social network analysis (SNA) is a methodology based on the study of relationships amongst social entities and on the patterns and implications of these relationships (Wasserman and Faust, 1994). SNA has been applied extensively in human epidemiology to improve understanding on diseases transmitted especially through sexual contacts (Liljeros et al., 2003; Rothenberg, 2003; Doherty et al., 2005). Within the veterinary domain, in the wake of the 2001 FMD epidemic in the UK, there has been a growing body of research employing SNA tools for the analysis of livestock movement data (Shirley and Rushton, 2005; Bigras-Poulin et al., 2006; Kao et al., 2006; Kiss et al., 2006a; Bigras-Poulin et al., 2007; Natale et al., 2009; Bajardi et al., 2011; Rautureau et al., 2011). Live cattle trade can be represented as a network of *nodes* (animal holdings/premises) whose interaction is mediated by *arcs* (animal movements). The arcs of the network serve as paths for the diffusion of diseases in the network (Dubé et al., 2009). For readability purposes, a glossary of the network terms used in the present study is provided in Table 1. For extensive reviews of network terminology as applied to the veterinary field, see Dubé et al. (2009) and Martinez-Lopez et al. (2009).

The study of the networks of livestock movements provides a means for understanding the dynamics of diseases on the networks as well as a method for defining effective control measures (Dubé et al., 2009). Amongst livestock networks, the *centrality* of nodes has been used as an important indicator of holdings that are influential in mediating the flow of animals in the network. These highly connected premises (hubs) have been considered to be at risk of becoming infected and transmitting the infection to others (Shirley and Rushton, 2005). This is a distinctive characteristic of *scale-free* networks, in which only a few nodes in the networks monopolise most of the contacts whereas most nodes have few contacts (Albert et al., 1999; Barabási and Albert, 1999). Individuals that are added to the network have an inclination to preferentially attach to those that already have a large number of contacts – a *disassortative* mixing pattern. Therefore, disease control strategies that target hubs in such networks will be more effective than those applied on randomly selected nodes (May and Llyod, 2001; Kiss et al., 2006a). Analyses of livestock networks has revealed a coexistence of both scale-free and *small-world* properties (Bigras-Poulin et al., 2007; Dubé et al., 2011), an indication that these categories are not mutually exclusive and should be collectively assessed in analyses of

livestock networks. Network cohesiveness refers to the level of connectedness in the network, and determines how fast and wide an infection can spread. Sparse networks allow limited local transmission, although infection can spread to the rest of the network in relatively few time steps (Shirley and Rushton, 2005). Successful application of disease control strategies can be determined by an assessment of the structural vulnerability of a network (Rautureau et al., 2011). The vulnerability of a network is the response observed when nodes are removed (Albert et al., 2000). Scale-free networks are often highly susceptible to targeted node removal, but tolerant to random deletion. Fragmentation of the network following node removal may impact the speed and extent of disease spread (Solé and Montoya, 2001).

Previous network studies have largely focussed on estimating snapshot network properties based on static networks (created by aggregating data over defined time scales) without systematically evaluating the evolution of these features over time (Bajardi et al., 2011). If indeed these properties were truly dynamical, control measures recommended for previous time points would be essentially ineffective for subsequent snapshots of time owing to the temporal evolution of the network. However, by computing distributions of these properties based on successive static networks, it is possible to assess the stability of the network *topology* over time (Bajardi et al., 2011).

The objective of this study was to characterise the network of Danish cattle movements over a 10-year period from 2000 to 2009 with a view to understanding: (1) cohesiveness of the network, (2) influential holdings and (3) structural vulnerability of the network. Since the goal of this work is to understand the underlying contact pattern of the Danish cattle herd population and its implication for disease control, the analysis is not constrained to a specific disease. Rather, we seek to ensure applicability of the results to a range of pathogens spread primarily through animal movements. This work thus forms the basis for informing future decisions aimed at optimising methods for controlling contagious pathogens, for instance, imposing movement restrictions on infected herds in an effort to contain the spread of *Streptococcus agalactiae* (*S. agalactiae*) within the Danish cattle herd population.

Table 1

A description of network terms as used in the present study.

Term	Definition	Reference
Arc	Directed link between 2 premises in a network	Wasserman and Faust (1994)
Assortativity	Correlation between the degrees of linked premises.	Newman (2003)
Average path length	Shortest path between 2 premises averaged over all pairs of premises in the network.	Watts and Strogatz (1998)
Betweenness	The frequency a premise is in the shortest path between pairs of network premises.	Freeman (1978)
Centrality	Importance of premises in the network.	Wasserman and Faust (1994)
Closeness	The inverse of farness – sum of shortest distances to all other premises in the network.	Wasserman and Faust (1994)
Clustering coefficient	Proportion of a premise's neighbours who are also neighbours of one another.	Watts and Strogatz (1998)
Component	Maximally connected sub-region of a network in which all premises are either directly or indirectly linked.	Robinson et al. (2007)
Degree	The number of links a premise has to other premises. For directed networks, the in-degree is the number of incoming links whereas the out-degree is the number of outgoing links.	Wasserman and Faust (1994)
Density	Sum of the number of all links divided by the number of possible links in the network.	Wasserman and Faust (1994)
Disassortative	Mixing pattern in which low degree premises tend to attach to high degree premises.	Newman (2002)
Infection chain	Number of premises that are reachable directly, in a single step, or indirectly, through other premises.	Dubé et al. (2008)
Giant strong component (GSC)	Largest component in a directed network in which all premises are mutually accessible following the direction of links in the network.	Kao et al. (2006)
Giant weak component (GWC)	Largest component in an undirected network in which all premises are linked without considering the direction of the links.	Kao et al. (2006)
Node	Smallest unit of concern in network analysis.	Wasserman and Faust (1994)
Random	Network characterised by lack of heterogeneity in the number of contacts and a lack of clustering.	Newman et al. (2002)
Scale-free	Network in which the out-and in-degree distributions fit a power law distribution.	Barabasi and Bonabeau (2003)
Small-world	Network characterised by high clustering and short path length.	Watts and Strogatz (1998)
Topology	Arrangement of nodes and arcs in a network.	Watts and Strogatz (1998)

2. Materials and Methods

2.1. Data

As part of the EU, Denmark maintains an elaborate electronic cattle register (the Central Herd Register, Danish Veterinary and Food Administration, Glostrup, Denmark), which captures data on all daily cattle movements within the country. This serves as a key traceability system, the basis for which infective animal movements can be easily trailed. For the purpose of this study, data on all cattle movements extending over the period of interest (2000 – 2009 inclusive) were extracted from the register. Each movement record detailed the unique identifier of the animal involved, its movement date, the identity of its source and destination premises, type of the premises (i.e. beef, dairy, breeder, dealer, market, animal show, communal pasture, abattoir and animal hospital) and the premise-specific georeferences. In Denmark, by definition a dairy farm is one that delivers milk to the factory. A non-milk-producing dairy farm (non-dairy) is thereby, broadly speaking, considered to be beef. Beef to beef movements could represent movements of calves to feedlot. On the one hand, beef to dairy movements could represent movements of dairy calves (non-dairy) to dairy farms. On the other hand, dairy to beef movements could correspond to translocations of dairy bull calves to beef farms. A breeder farm is an insemination station and by way of example, beef to breeder movements could represent the transport of beef bulls to the station for semen collection and back. Markets are centres where animals are received destined for either slaughter i.e. slaughter market or for sale to other farms such as beef. Notably, movements to abattoirs were excluded from the analyses as they represent end points in the movement chain and as such have negligible role in the onward transmission of infection.

2.2. Descriptive analysis

Standard descriptive statistics (proportions, medians and graphs) for the individual animal movements and type of premises were computed using Stata software (Stata version 11.2, Stata Corporation, College Station TX, USA). Euclidean distances of movement between the source and destination premises were estimated based on the premises' geo-coordinates.

2.3. Network analysis

The cattle trade system was represented as a network comprising a set of nodes (premises) and arcs formed by individual cattle displacements between premises. Considering that the direction of movement was known, the resulting network was directed. Static networks were created based on individual cattle movements between source and destination premises in each month resulting in 120 networks. Although daily networks could be considered choice alternatives for capturing the intrinsic dynamical nature of the system (Bajardi et al., 2011), the rationale for selecting a monthly timescale was based on two arguments: (1) the need to strike a balance between having sufficient number of nodes at any given time window and the number of networks to be analysed and (2) the fact that it was considered a reasonable duration, during which an infection could spread silently. Besides, Keeling and Eames (2005) contend that using larger time windows may not be problematic if changes in connections amongst nodes are slow relative to the timescale of the pathogen under consideration. In such cases, the network structure remains relatively invariant during the epidemic process.

The cohesiveness of the networks was evaluated by computing the parameters: *density*, *clustering coefficient* and *average path length* and plotting their distributions against time. Trends in the cohesion measures were

tested using the Mann-Kendall test – a non-parametric test that evaluates the null hypothesis of no trend in time series data.

Centrality measures, namely: *betweenness*, *closeness* and *degree* were also estimated. In order to identify influential holdings in the networks, the median centrality values for each premise type were calculated in each month, after which overall medians for the entire duration of study were obtained. Premise types with the highest of these values were deemed the most central.

To determine whether the networks were scale-free, fits to power law behaviour of in-and out-degree distributions were computed. Mathematically, a quantity k obeys a power law if it is drawn from a probability distribution of the kind: $p(k) \sim k^{-\alpha}$, where α is a constant parameter of the distribution referred to as the exponent. This parameter characteristically lies in the range $2 < \alpha < 3$ (Clauset et al., 2009). The degree distributions were visualised on log-log plots with a straight line on such plots being suggestive of a power law distribution (Barabási, 2003). We also assessed whether the networks displayed small-world properties. This was achieved by generating *random* networks with similar numbers of nodes and arcs as the observed networks using the Erdös-Rényi model (Erdös and Rényi, 1960). The networks were considered small-world if they met the criteria laid out by Dubé et al. (2011): the clustering coefficients in the observed networks should be at least 20 times greater than in the random networks, and that the average path lengths for the two networks should be relatively similar or shorter in the observed networks. Network *assortativity* was also estimated. The assortativity coefficient is a useful indicator of the susceptibility of networks to either random or targeted node removal procedures (Newman, 2002).

Network *component* sizes: *giant strong* and *weak component* sizes (GSC and GWC, respectively) were also computed and the distributions of their sizes plotted against time. Notably, the monthly sizes of GSCs and GWCs were expressed as proportions of the total number of premise types in the given month to ensure temporal comparisons. As with the cohesion measures, we tested for trends in the distributions of GSC and GWC. In order to assess the vulnerability of the networks, the most central premises in the global networks were removed. Subsequently, the sizes of the GSCs were recalculated and their distributions shown over time. Network analyses were carried out using the Igraph package (Csardi and Nepusz, 2006) for R software (R Development Core Team, 2012).

3. Results

3.1. Description of the data

A total of 50,494 premises were registered in the database over the 10-year span. Beef accounted for the largest proportion of the premises (75.5%, n = 38,126) followed by dairy at 23.4% (n = 11,808). In total, 3,013,452 cattle were involved in 4,204,895 individual movements. Of the number of cattle, 75.9% (n = 2,287,339) were moved once during the period. For movements involving batches of animals (a batch movement defined as movement of a group of animals between 2 premises on the same day), 690,346 batches were transported (representing 16.4% of total movements). A majority of the movements occurred between beef and dairy premises. Moreover, beef premises were the highest recipients of the movements (57.3%, n = 2,409,122), while dairy premises represented the highest donors (67.5%, n = 2,836,830) (Table 2). There was

a steady increase in the number of movements during the 10-year period with the lowest and highest displacements taking place in March 2001 (month 15) and May 2008 (month 113) respectively (Fig. 1). No movements took place to and from markets in March to June and August 2001 (months 15 to 18 and 20, respectively). The median distance over which cattle were moved was 15.3 km (range: 0 – 444.5 km).

3.2. Characteristics of the networks

The median numbers of nodes and arcs for the monthly networks were 7,031 and 6,764 respectively (Table 3). The networks were sparsely connected as indicated by the consistently low, but fluctuant distributions of values of the cohesion parameters (Fig. 2a – c). Regardless of the time window, the path lengths were short with any linked pair of premises being on average separated by a sequence of 4 arcs/steps, which corresponds to 3 intermediary nodes. A sharp rise in the clustering coefficient coupled with a steep decline in the average path length was observed in March to April 2001 (Fig.2b – c) (months 15 and 16, respectively). In these months, the clustering coefficients for the observed networks were infinity and 31 times greater than those for the corresponding random networks (for March and April respectively), whereas the average path lengths were 2.6 and 2.8 times longer (for the same months) in the random networks compared to the observed networks; observations consistent with small-world properties (Table 4). Additionally, pockets of small-world characteristics were detected in 24 other networks spanning October 2006 to December 2009. Both density and clustering coefficient measures showed increasing trends ($P < 0.001$) whereas no trend was observed for the average path length ($P = 0.89$). Values of the cohesion measures are summarised in Table 3. The networks were disassortative as given by the negative assortativity coefficients (Table 3); nodes had a higher tendency to connect to nodes with dissimilar degrees compared to those with similar degrees. The distribution of the assortativity measure was highly fluctuant (Fig. 2d). The in-and out-degree distributions approximated power law behaviour as depicted by the linear shape on the log-log plot (Fig. 2e). The median values of the exponents were 2.18 and 2.03 for the in-and out-degree respectively (Table 3). The distributions of these exponents were generally stable over time (Fig. 2f). Within the global networks, the most influential holdings were markets having the highest betweenness, closeness and degree scores (Table 5).

The frequency distribution of the GSC and GWC sizes is shown in Fig. 3. As was the case with the cohesion parameters, their patterns were highly fluctuant and marked by a distinguishable decline in March to April 2001 (months 15 and 16, respectively). Approximately 0.38% and 10.76% of nodes were included in the GSCs and GWCs respectively (Table 3). An increasing trend for the distribution of GWC was detected ($P < 0.001$), but no trend was noticeable for the GSC distribution ($P = 0.67$). Following the removal of markets from the global networks, disappearance of the GSCs was noted (Fig. 3b).

Table 2

The number of cattle movements in Denmark by type of source and destination premises during the period 2000 – 2009.

Type of source premise	Type of destination premise (%)								
	Beef	Dairy	Breeder	Dealer	Market	Animal show	Communal pasture	Animal hospital	Total (%)
Beef	490,469	324,221	2,671	26,744	72,762	30,047	23,345	35	970,294
	(50.6)	(33.4)	(0.3)	(2.8)	(7.5)	(3.1)	(2.4)	(0)	(23.0)
Dairy	1,741,145	851,495	2,613	45,523	76,334	70,472	48,901	347	2,836,830
	(61.4)	(30.0)	(0.1)	(1.6)	(2.7)	(2.5)	(1.7)	(0)	(67.5)
Breeder	2,433	38	8,795	0	0	202	0	0	11,468
	(21.2)	(0.3)	(76.7)	(0)	(0)	(1.8)	(0)	(0)	(0.3)
Dealer	35,579	19,580	3	1,691	35,579	69	83	0	92,584
	(38.4)	(21.2)	(0)	(1.8)	(38.4)	(0.1)	(0.1)	(0)	(2.2)
Market	101,997	55,698	0	26,094	6	0	0	0	183,795
	(55.5)	(30.3)	(0)	(14.2)	(0)	(0)	(0)	(0)	(4.4)
Animal	14,403	24,729	17	10	37	32	0	0	39,228
show	(36.7)	(63.0)	(0)	(0)	(0.1)	(0.1)	(0)	(0)	(0.9)
Communal	23,094	47,497	0	79	0	0	5	0	70,675
pasture	(32.7)	(67.2)	(0)	(0.1)	(0)	(0)	(0)	(0)	(1.7)
Animal	2	18	0	0	0	0	0	1	21 (0)
hospital	(9.5)	(85.7)	(0)	(0)	(0)	(0)	(0)	(4.8)	
Total (%)	2,409,122	1,323,276	14,099	100,141	184,718	100,822	72,334	383 (0)	4,204,895
	(57.3)	(31.5)	(0.3)	(2.4)	(4.4)	(2.4)	(1.7)		

Table 3

Descriptive parameters for the monthly networks of Danish cattle movements during the period 2000 – 2009.

Parameter	Median	Range
Nodes	7031	5284 – 10640
Arcs	6764	4225 – 10680
[a]Density	0.01	0.01 – 0.02
[b]Clustering coefficient	0.27	0.13 – 1.25
Average path length	4.28	1.39 – 8.76
Assortativity coefficient	-0.08	-0.14 – -0.04
Power law exponents		
α (in-degree)	2.18	2.10 – 2.25
α (out-degree)	2.03	1.97 – 2.15
[c]Component sizes		
GSC	0.38	0.01 – 2.52
GWC	10.76	1.01 – 15.69

[a, b, c]Expressed as percentages.

Table 4

Comparisons between observed and random networks for the months of March and April 2001.

Month	Nodes (Arcs)	Observed		Random	
		[a,b]Clustering coeff.	[c]Av. path length	Clustering coeff.	Av. path length
March	5586 (4225)	0.93	1.39	0	3.55
April	6175 (4757)	1.25	1.42	0.04	3.97

[a]Clustering coefficient.

[b]Expressed as a percentage.

[c]Average path length.

Table 5

Premise-specific median centrality values for the monthly networks of Danish cattle movements during the period 2000 – 2009.

	Betweenness score	Closeness score	Degree score
Premise	Median (range)	Median (range)	Median (range)
Beef	0 (0 – 0)	2.3×10^{-8} (9.2×10^{-9} – 6.4×10^{-8})	1.0 (1.0 – 1.0)
Dairy	0 (0 – 0)	4.5×10^{-8} (2.0×10^{-8} – 7.6×10^{-8})	1.0 (1.0 – 1.0)
Breeder	2.0 (0 – 30936.6)	3.5×10^{-8} (1.4×10^{-8} – 7.6×10^{-8})	3.5 (1.0 – 18.0)
Dealer	1391.5 (0 – 5138.0)	4.5×10^{-8} (2.0×10^{-8} – 7.6×10^{-8})	4.0 (2.0 – 6.0)
Market	278581.4 (0 – 1592960.0)	4.5×10^{-8} (2.0×10^{-8} – 7.6×10^{-8})	335.5 (1.0 – 532.0)
Animal show	0 (0 – 217435.3)	4.2×10^{-8} (1.6×10^{-8} – 7.6×10^{-8})	25.0 (1.0 – 156.0)
Communal pasture	0 (0 – 4.0)	2.8×10^{-8} (1.4×10^{-8} – 7.6×10^{-8})	2.0 (1.0 – 7.5)
Animal hospital	17.5 (0 – 30381.0)	3.9×10^{-8} (1.8×10^{-8} – 6.0×10^{-8})	9.5 (3.0 – 34.0)

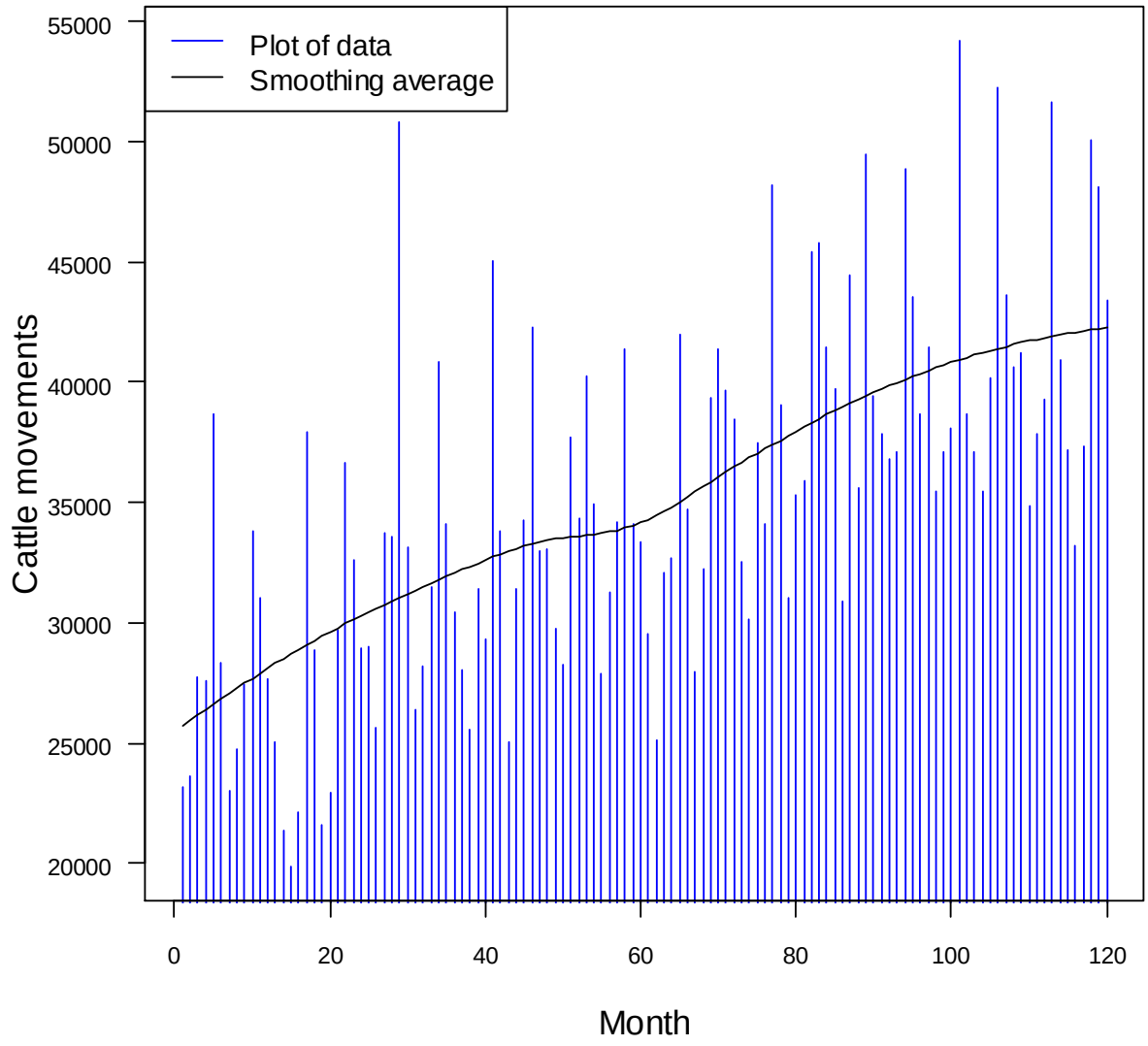

Fig. 1. Plot of the number of monthly cattle movements in Denmark during the period 2000 – 2009.

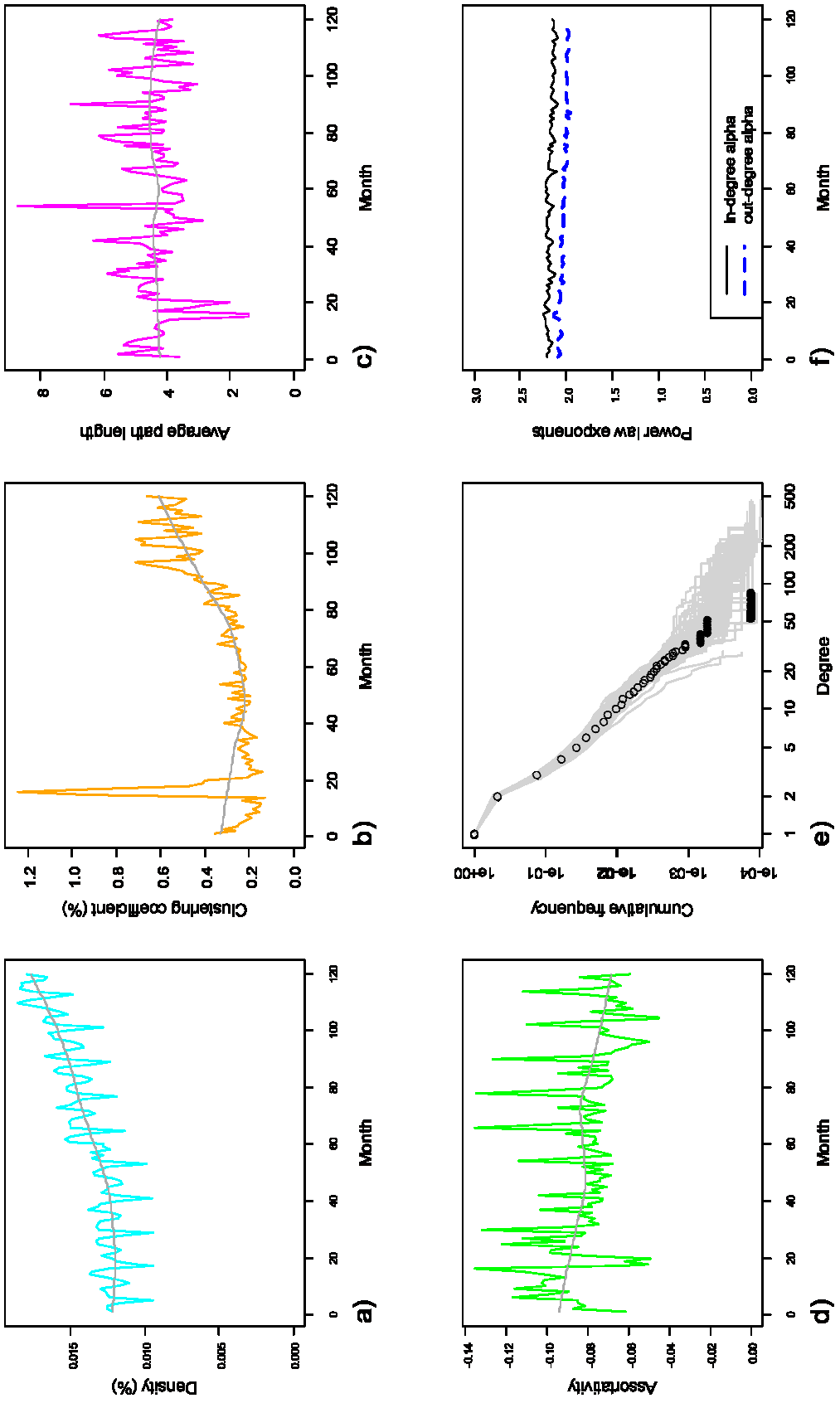

Fig. 2. Plots of the distributions of (a) density, (b) clustering coefficient, (c) average path length, (d) assortativity coefficient (with smoothing averages indicated by continuous dark grey lines), (e) in-degree distribution for January, 2000 (black) (underlain by the in-degree distributions for the other months [light grey]) and (f) power law exponents in the monthly networks of Danish cattle movements during the period 2000 – 2009.

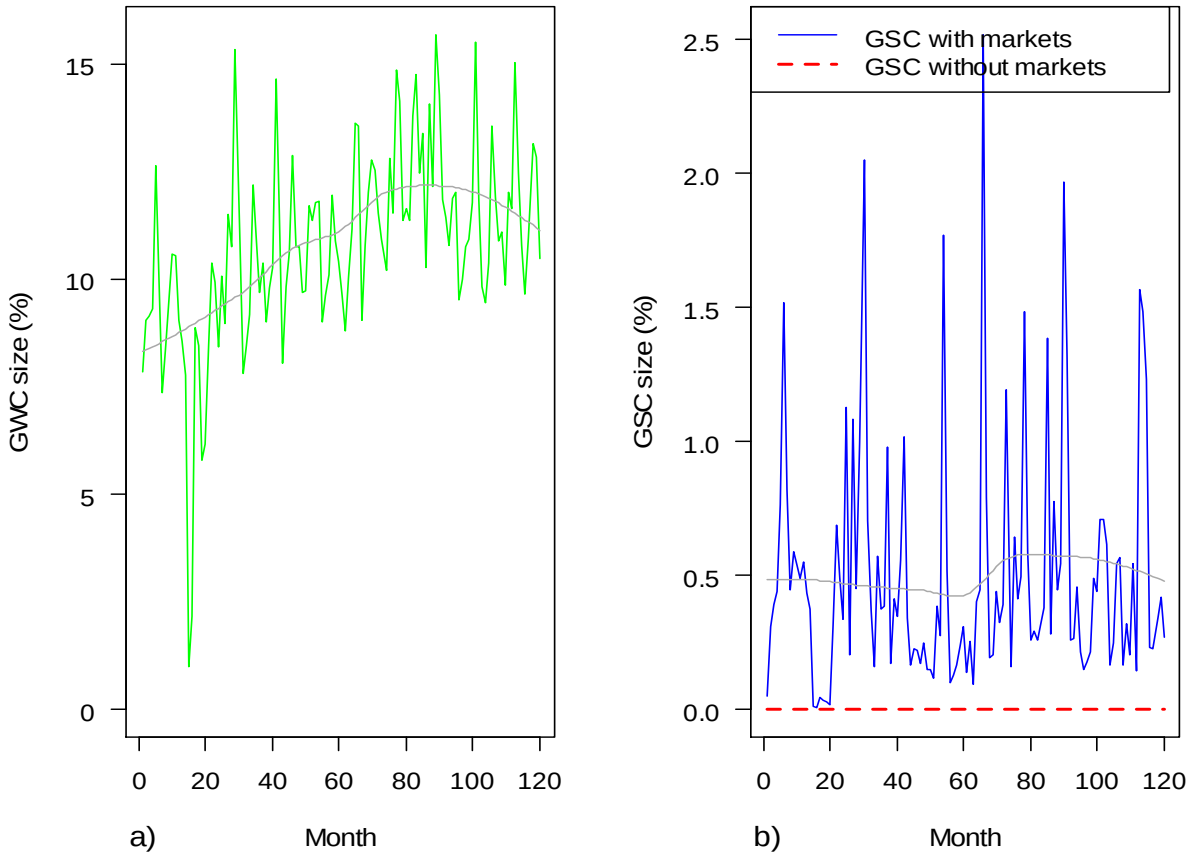

Fig. 3. Plots of the distributions of (a) giant weak component (GWC) and (b) giant strong component (GSC) (before and after removal of markets) in the monthly networks of Danish cattle movements during the period 2000 – 2009. Smoothing averages are indicated by continuous dark grey lines.

4. Discussion

Analysis of the longitudinal data has revealed that the network of Danish cattle movements was predominantly scale-free. However, it was punctuated by small-world properties in March to April 2001 as well as in 24 other months in the period October 2006 to December 2009. In particular, the appearance of these properties in March to April 2001 coincided with the occurrence of the 2001 FMD epidemic in the UK whose impact extended into much of the European livestock industry. In Denmark, as in other parts of Europe, the imposition of movement restrictions during the epidemic led to reorganisation of the network permitting the formation of local clusters with possibilities of long-distance connections. A high level of clustering coupled with short path lengths, as is found in small-world networks, allows most infection to spread locally although spread to topologically distant clusters within the network is also probable (Watts and Strogatz, 1998). An explanation for the presence of these properties during the period October 2006 to December 2009 is yet to be established. The coexistence of both scale-free and small-world properties in the network of Danish cattle movements is consistent with previous observations of livestock movement networks (Bigras-Poulin et al., 2006; Bigras-Poulin et al., 2007; Natale et al., 2009).

The clustering coefficient and average path length values reported in this study fall within the range of observed values for other scale-free livestock networks: 0.4%; 4.97 (Rautureau et al., 2011) and 0.4%; 7.82 (Natale et al., 2009) for clustering coefficient and average path length respectively. The small values of these parameters, which illustrate a sparsely connected network, suggest that an epidemic is likely to spread minimally locally, but rapidly through the network (Keeling and Eames, 2005). However, with the increasing trend in density and clustering coefficient, a reflection of increasing connectedness in the network, future infection spread is likely to be more localised. Markets were shown to be the most influential holdings in the global networks. Analysis of an Italian cattle network (Natale et al., 2009; Bajardi et al., 2011) showed that markets dominated the global networks, while within British and French networks, dealers and markets were the key players (Christley et al., 2005; Rautureau et al., 2011). The presence of these highly connected premises in a largely sparsely connected network is a distinguishing mark of scale-free networks (Scholz, 2010). Rautureau et al. (2011) demonstrated that clustering coefficients within GSCs seemed inversely related to node degrees with a roughly linear relationship on a log-log scale. By contrast, low degree nodes were highly involved in dense sub-networks. Because these hubs have been considered to exhibit super-spreader behaviour, their presence in scale-free networks renders the networks prone to spread and persistence of infections (Keeling and Eames, 2005). Thus, in such networks infectious agents possessing low transmissibility are capable of invading and causing large epidemics (Barabási, 2009). The linear trend in animal movements together with the increasing trend in the distribution of GWC (Fig. 1 and 3a, respectively) suggest a heightened risk of spread of contagious pathogens within the Danish cattle herd population; a hypothesis recently confirmed with respect to spread of *S. agalactiae* (Mweu et al., 2012).

The degree distributions in the present study were linear on a log-log scale with the median power law exponents lying within the acceptable range for power law distributions (range: 2 – 3)(Clauset et al., 2009). This finding concurs with previous observations in Denmark where the in- and out-degree exponent values of 2 and 1.46 respectively, were obtained (Bigras-Poulin et al., 2007). Similar values have been reported from analyses of British 2.1 (Christley et al., 2005), Italian 2.26 (Natale et al., 2009) and French 2.58 (Rautureau et al., 2011) cattle networks. The power law degree distributions noted here suggest heterogeneity in the number of contacts in the networks with hubs enjoying a disproportionately large share. This heterogeneity was partly attributable to the disassortative mixing pattern seen in the networks where low degree nodes had a tendency to preferentially attach to others with already a large number of contacts. The heterogeneous contact pattern explains why scale-free networks are especially resilient to disease control measures that mimic random node removal since a randomly selected node is likely to be weakly connected and its deletion would have little impact on the structural integrity of the network (Albert et al., 2000).

In this study, following the removal of markets from the networks, disappearance of GSCs was noted. This finding illustrates the vulnerability of the Danish cattle network to targeted application of control measures and further underscores the importance of focussing control efforts on highly connected premises, which could potentially eliminate a possible epidemic. This observation corroborates findings from past studies on livestock networks (Kao et al., 2006; Kiss et al., 2006b; Rautureau et al., 2011). In particular, Rautureau et al. (2011) investigated targeting procedures based on holding type and node centrality measures and showed that the latter was a more effective strategy in prompting GSC disappearance. Indisputably, regardless of the

timescale, shutting down markets ought to be considered a significant step in containing the spread of an epidemic within the Danish cattle herd population.

Availability of longitudinal data has afforded a unique opportunity to evaluate whether the Danish cattle movement network has evolved over the 10-year span based on the estimation of snapshot network properties. Except for the pockets of small-world properties, the topology of the network was predominantly scale-free with markets serving as hubs. This result may come as a relief to policymakers in the sense that control measures deemed efficacious in past timescales may still prove successful in containing epidemics on the network in subsequent periods. However, it is vital that formulation of control strategies for an epidemic be based on information available at premise-level (where a consistent pattern was evident) but not at individual holding (node) level where centralities were highly variable across the study period. This view supports that of Bajardi et al. (2011), who demonstrated the coexistence of stationary statistical distributions with dynamical node-level properties for the network of Italian cattle movements in 2007 and accordingly, cautioned against making inferences for subsequent time points based on node-level information from previous time steps.

For comparability purposes, we also assessed the effect of using longer aggregation time scales (i.e. quarterly, semiannual and annual windows) on the topology of the network. Overall, although the networks were increasingly larger with increasing size of the time windows, a gradual masking of detail was evident in the network properties distributions with clearer trends in connectivity and notable widening departure of the networks from scale-free behaviour. However, irrespective of the time scale, markets dominated the networks and their removal was invariably followed by disappearance of GSCs. Indeed, a careful choice of the time window is necessary to reduce the likelihood of spurious inferences. The size of the window is not only determined by the dynamics of the pathogen under investigation but also by the level at which the analysis is intended. When the unit of concern is the individual animal (within-herd analysis), owing to the close proximity amongst herd mates, a shorter time scale may be the optimal choice. However, longer time scales may suffice in the case of a herd-level analysis in situations where between-herd movements are fairly infrequent.

A potential bias inherent in the estimates of network properties obtained in this study stems from an inability of the estimation algorithm to account for arc weights. Analysis of weighted networks is complex and, although several methods for their analyses have been proposed, they are still at their infancy and often incompatible with large networks (Barrat et al., 2004; Opsahl et al., 2010). In a bid to circumvent this challenge, Kao et al. (2006) created an "epidemiological network" based on truly infectious links where the probability of transmission across a link (p_{ij}) joining nodes i and j was weighted by the characteristics of i and j. Although this approach may seem promising, an obvious drawback to its use arises from the fact that for most contagious pathogens p is often quite small and thus, for some periods stochastically, zero infectious links might occur impeding further analysis. Furthermore, for the analysis of data extending over several years, temporal comparisons may be illogical given that the networks generated would be dependent on the transmission probability, which itself may be time-dependent.

Markets, dealers and communal pastures represent holdings where animals are held transiently before being shipped to other premises. It is therefore expected that the number of movements to and from these premises should be reciprocal. In these data, the incoming movements exceeded the outgoing movements for these premises; a discrepancy that could be attributable to animals being sent to slaughter.

The GSCs and GWCs have previously been used not only to evaluate the structural vulnerability of networks (Rautureau et al., 2011) but also to estimate potential epidemic sizes (Kao et al., 2006; Kiss et al., 2006b). However, their usefulness in providing reliable estimates of epidemic sizes has recently been brought to question due to their failure to account for the time sequence or direction of animal movements between holdings (Dubé et al., 2008, 2011). A rather more robust method - *infection chain* analysis - is being advocated as a suitable alternative for achieving this goal (Dubé et al., 2011; Nöremark et al., 2011).

Although this study strictly relates to cattle movements, a possibly worthwhile undertaking would have been to perform a joint cattle-swine network analysis with the goal of establishing whether the resulting networks differ and thereby exhibit distinct implications for disease control. Logistically speaking, this was infeasible in the current study given the length of the analysis period under consideration. Nonetheless, the results of an analysis of the Danish swine movement network over an eight-month period (September 30 2002 to May 22 2003) by Bigras-Poulin et al. (2007) revealed the network as scale-free and possessing a small-world property. This finding is consistent with observations made in our study.

By limiting the analysis to movement data, we were unable to capture other non-movement-related transmission pathways such as air-borne spread, which are characteristic of many contagious pathogens. With this in mind, imposing movement restrictions such as market closure during epidemics should only be considered as a preliminary strategy, which if supplemented with other measures e.g. cleaning and disinfection of premises in the case of FMD could lead to the containment of an epidemic. Shirley and Rushton (2005) noted that most of the infectious events associated with the 2001 UK FMD epidemic occurred subsequent to the movement ban mainly related to local spread. Interestingly though, they also reported that the number of cases arising from each infectious premise following the ban was low compared to the period preceeding the ban; an indication of the effectiveness of targeted control measures.

This work has provided a useful insight into the contact pattern of the Danish cattle herd population that deviates from the random-mixing assumption in classical epidemiological modelling. Failure to account for the population heterogeneity in simulation modelling could potentially result in a misestimation of the effectiveness of control measures (Webb and Sauter-Louis, 2002).

5. Conclusion

Network analysis of Danish cattle movements during the period 2000 – 2009 has revealed the primarily scale-free nature of the network, which was however interrupted by small-world properties in March to April 2001 as well as in 24 other months during the period October 2006 to December 2009. The network was sparsely connected with markets being the key influential holdings. Vulnerability of the network to measures that target hubs underlines the importance of focussing control efforts on highly connected premises such as

closure of markets during epidemics. The findings from this study suggest that understanding the contact structure of a susceptible population is advisable before embarking on any strategy for disease control. As the influence of individual holdings is subject to temporal fluctuations, definition of control strategies should instead be based at the premise level. Outcomes of this work can be implemented in future simulation models of the spread and control of contagious pathogens such as *S. agalactiae* in the population of Danish cattle herds.

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CHAPTER 5:

SPATIOTEMPORAL PATTERNS, ANNUAL BASELINE AND MOVEMENT-RELATED INCIDENCE OF *STREPTOCOCCUS AGALACTIAE* INFECTION IN DANISH DAIRY HERDS: 2000 – 2009

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Abstract

Several decades after the inception of the five-point plan for the control of contagious mastitis pathogens, *Streptococcus agalactiae* (*S. agalactiae*) persists as a fundamental threat to the dairy industry in many countries. A better understanding of the relative importance of within-and between-herd sources of new herd infections coupled with the spatiotemporal distribution of the infection, may aid in effective targeting of control efforts. Thus, the objectives of this study were: (1) to describe the spatiotemporal patterns of infection with *S. agalactiae* in the population of Danish dairy herds from 2000 – 2009 and (2) to estimate the annual herd-level baseline and movement-related incidence risks of *S. agalactiae* infection over the 10-year period.

The analysis involved registry data on bacteriological culture of all bulk tank milk samples collected as part of the mandatory Danish *S. agalactiae* surveillance scheme as well as live cattle movements into dairy herds during the specified 10-year period. The results indicated that the predicted risk of a herd becoming infected with *S. agalactiae* varied spatiotemporally; the risk being more homogeneous and higher in the period after 2005. Additionally, the annual baseline risks yielded significant yet distinctive patterns before and after 2005 – the risk of infection being higher in the latter phase. By contrast, the annual movement-related risks revealed a non-significant pattern over the 10-year period. There was neither evidence for spatial clustering of cases relative to the population of herds at risk nor spatial dependency between herds. Nevertheless, the results signal a need to beef up within-herd biosecurity in order to reduce the risk of new herd infections.

Keywords: Biosecurity; Cattle; Incidence; Spatiotemporal; *Streptococcus agalactiae*

1. Introduction

Streptococcus agalactiae (*S. agalactiae*) is a contagious pathogen that exerts deleterious effects on bovine udder health, milk quality and productivity (Keefe, 1997). Consequently, in a quest to maintain production efficiency at the herd level, a justification for the deployment of resources aimed at preventing the introduction and subsequent within-herd spread of *S. agalactiae* is provided. In Denmark, a national surveillance and control scheme for *S. agalactiae* has been operational since 1966 (Anon., 1966). This was initiated against a backdrop of worrisome infection prevalences in the 1950s and 60s (Andersen et al., 2003). The control programme (with compulsory surveillance) entailed a rigorous combined application of within-herd sanitary and hygienic measures coupled with a prohibition on the sale of cows and pregnant heifers from infected herds (Andersen et al., 2003). Thanks to these concerted efforts, the herd-level prevalence of *S. agalactiae* declined appreciably from 30 to 40% in 1950 (Anon., 1981) to approximately 2% in 1992, with a 1 – 2% incidence of herd infections (Anon., 1980 – 1992). However, since 2000 an increasing trend in both the herd-level prevalence and incidence of *S. agalactiae* has been reported suggesting a re-emergence of the pathogen in the Danish dairy herd population (Mweu et al., 2012a).

New herd infections with *S. agalactiae* can arise from internal (within-herd) and/or external (between-herd) factors. Of the internal factors, there is mounting evidence implicating humans as potential sources of infection for cattle (Jensen, 1982; Zadoks and Schukken, 2006; Zadoks et al., 2011). Notable personnel included in this internal category are farmers and relief milkers. Asymptomatic human carriage of *S. agalactiae* occurs in the urogenital and gastrointestinal tracts as well as the skin (Van der Mee-Marquet et al., 2008) and is frequent in young adults (20-40%) and the elderly (22%) (Manning et al., 2004; 2008; Edwards and Baker, 2005). A probable route of infection transfer to the cow is via hands during milking (Edmondson, 2011). Recently, work by Zadoks et al. (2011) involving multi-locus sequence typing of 111 isolates collected from a 2009 Danish bulk tank milk survey demonstrated the commonest *S. agalactiae* strains to be sequence types (ST) 1 (28%) and ST 23 (23%), which were previously primarily associated with human infections. Bovine strains are also recognisable, with introduction of new possibly infected animals into susceptible herds having been shown to constitute an external risk (Agger et al., 1994). This is particularly true for large herds with less stringent biosecurity measures whose pursuit for rapid expansion may favour acquisition as opposed to internal growth (Barkema et al., 2009). Spatially-varying risk factors such as sharing of farm equipment may serve as additional avenues for the between-herd spread of *S. agalactiae*. Revisiting the human argument: dairy herds are visited by a variety of personnel who establish variable degrees of physical contact with cattle. Along with relief milkers (who likewise pose a within-herd risk), veterinarians and inseminators display varying geographical scales of operation stretching from local to regional, which in turn may influence the range of the spatial spread of *S. agalactiae*. Regrettably, movement patterns of these persons are seldom recorded precluding an evaluation of their relative roles in determining new herd infections. Importantly, unlike other streptococci, *S. agalactiae* is highly host-adapted and no environmental reservoirs have yet been identified (Keefe, 1997; Manning et al., 2010).

Even though the annual trend in the pooled herd-level incidence of *S. agalactiae* has been estimated (Mweu et al., 2012a), a supplementary undertaking seeking to disentangle the relative importance of within-and between- herd sources of new herd infections is worthwhile. In particular, availability of cattle movement data

on individual herds can be critical in highlighting the possible role of animal introductions in driving *S. agalactiae* infections. Illustratively, by including exposure to animal introductions as a fixed effect in logistic regression analysis, the incidence risk attributable to these movements can be quantified. The risk in the unexposed herds is thus deemed representative of the 'baseline' risk in the general population of herds (Dohoo et al., 2009). In the absence of observable spatial dependency following semivariogram analysis of the logistic regression residuals, it is acknowledged that after accounting for the effects of known between-herd factors in the model, the source of the remaining risk rests in within-herd factors rather than in spatially-varying ones (Pfeiffer et al., 2008; Stevens et al., 2009). An understanding of the magnitude of the respective risks can be instrumental in guiding *S. agalactiae* policy formulation, thus ensuring effective targeting of control efforts.

A crucial step towards gaining insight into the epidemiology of a contagious pathogen involves investigating its geographical distribution - and if available data span a given time range – its associated temporal aspects. This serves the purpose of facilitating causal hypotheses generation, after which these can be formally tested (Berke, 2005). For instance, Fenton et al. (2009) investigated both large-and small-scale spatial and temporal patterns of infection in dairy herds with *Salmonella enterica* serovars. The number of cases arising within a defined distance and time period of an index case was found to be higher than expected. This provided evidence for spatiotemporal clustering suggesting the existence of either a contagious process or locally-acting environmental factors which increased the risk of infection. To date and to the best of our knowledge, there are no published studies examining the spatiotemporal epidemiology of *S. agalactiae*. Hence, the objectives of this study were: (1) to describe the spatiotemporal patterns of infection with *S. agalactiae* in the population of Danish dairy herds from 2000 – 2009 and (2) to estimate the annual herd-level baseline and movement-related incidence risks of *S. agalactiae* infection over the 10-year period.

2. Materials and methods

2.1. Data

Data for the present study comprised *S. agalactiae* surveillance and live cattle movement data extending over the period 1999 – 2009. The surveillance data were extracted from the Danish Cattle Database, which holds information on bacteriological culture of all bulk tank milk (BTM) samples collected as part of the mandatory Danish *S. agalactiae* surveillance scheme. The scheme entails an annual collection of BTM samples by truck drivers during milk collection, following which the samples are stored on ice. Within 24 h, they are sent to Eurofins laboratory (Holstebro, Denmark) for processing. Bacteriological culture of the samples follows the National Mastitis Council (1999) standards. Besides the test outcome, the herd-specific geo-coordinates (recorded as UTM EUREF89, zone 32 coordinates) and the test date are specified. The cattle movement data were extracted from the Central Herd Register (Danish Veterinary and Food Administration, Glostrup, Denmark). The register captures data on all daily cattle movements within the country. Each movement record details the unique identifier of the animal involved, its movement date, the identity of the source and destination premises and type of the premises (i.e. beef, dairy, breeder, dealer, market, animal show, communal pasture and animal hospital). A description of the specific premise types is given elsewhere (Mweu et al., 2013).

With regards to the surveillance data, it is noteworthy that repeat testing was effected for selected herds in certain years especially if the first screening result was positive. Therefore, to ensure consistency with the rest of the data, only the initial test outcome for a given herd in a particular year was used to classify herds. By definition, a case was a herd from which *S. agalactiae* was cultured from its BTM sample; a non-case being otherwise defined. To generate an incidence dataset, cases were considered new in a given year only if they had been non-cases in the preceding year. As for the movement data, cattle translocations between source and destination premises in the year prior to each BTM survey were aggregated to give the number cattle transferred to specific premises. This was done to ensure a temporal sequence between any probable exposure associated with the importations and the risk of infection with the pathogen. Granted that the interest of the present study lay squarely in dairy herds, only movements to dairy herds were retained. Further, because movements between holdings that were registered under the same owner were also recorded, these were excluded since they were deemed not to pose an external risk. Subsequently, a single dataset combining the incidence and movement data was created.

2.2 Spatial and statistical analysis

For each herd in a given year, the time (in months) between screenings was computed and graphed to display the distribution of year-specific study periods.

2.2.1 Actual risk surfaces

In order to facilitate visualisation of the spatial distribution of cases and non-cases, a kernel smoothing technique was applied to the location of cases and non-cases in each year. Generation of the year-specific kernel density surfaces was based on the use of a quartic approximation of a true Gaussian kernel function and a common case-noncase fixed bandwidth, computed via a leave-one-out least squares cross-validation approach (Rudemo, 1982; Bowman, 1984) implemented in the *sparr* package (Davies et al., 2011) for R software (R Development Core Team, 2013). Arguably, the choice of the appropriate kernel function is comparably of less importance than the size of the bandwidth, with larger bandwidths yielding smoother surfaces (Berke, 2005). Considering that the computed bandwidths were year-specific, to permit temporal comparisons, a calculated median bandwidth value of 19 km was used. As there is not yet a mathematical algorithm developed to compute grid cell sizes, Pfeiffer et al. (2008) contend that the choice of an optimal grid cell size should instead stem from a presentational, biological and numerical perspective. Preferably, grid sizes ought to be larger than the geographical extent of the biological unit of interest. Given an average Danish farm size of 0.57 km^2 in 2004 (Levin et al., 2006), output grid cell sizes of 1 km^2 were utilised.

To correct for the spatial distribution of the underlying population of herds at risk, risk maps for each year were created by dividing the kernel density surfaces for cases and the population (given by summing case and non-case densities) in each year. The resulting risk surface provided an estimate of the probability of a herd contracting an infection with *S. agalactiae* at a specified location in a particular year (Bowman and Azzalini, 1997). The kernel density estimation was accomplished using the Spatial Analyst Extension available in ArcGIS 10.1 (ESRI, Redlands, CA, USA).

2.2.2 Spatial clustering

Evidence for spatial aggregation of cases over and above that of the population of all herds and scale of the distances over which clustering (if present) occurred, was investigated by means of the inhomogeneous *K*-function implemented in the *spatstat* package (Baddeley and Turner, 2005) for R software. The inhomogeneous *K*-function is a non-stationary analogue of the standard *K*-function (Ripley, 1976), which assesses the presence of clustering in spatial point processes after allowing for spatial heterogeneity in the underlying spatial distribution (Baddeley et al., 2000). Monte-Carlo randomisation with 499 simulations was used to randomly permute the location of cases and the entire population of herds at risk in each year. The 95% confidence bounds of these permutations were plotted together with the observed difference functions.

2.2.3 Baseline and movement-related risks

Initially, herds were aggregated into four geographical regions comprising: the eastern Danish islands (including Bornholm, Zealand and Funen), South, Mid and North Jutland. In order to estimate the annual baseline and movement-related incidence risks, year-specific mixed-effects logistic regression models were fitted to the data. The primary fixed effect in the models was whether or not a movement had taken place prior to screening in a particular year. Moreover, as estimates of the annual baseline risks could differ by virtue of study period differences, to obtain 12-months equivalent baseline risks and hence allow for temporal comparability, the study period variable was included in the models centred at 12 months. The variable region was included in the models as a random effect to account for first-order (large-scale) spatial effects. First-order effects describe the variation in the mean value of a process in space (Ripley, 1981). The corresponding equation for the year-specific models can be expressed as follows:

$$\text{logit}\,(P_i) = \beta_0\,(\text{intercept}) + \beta\,(\text{movement})_i + \beta\,(\text{study period})_i + \mu_{\text{region}(i)}$$

where P_i is the probability of the i^{th} herd becoming infected with *S. agalactiae* in a given year, β_is are the regression coefficients associated with the fixed effect variables for the i^{th} herd and $\mu_{\text{region}(i)}$ is the random effect of the region containing herd i, assumed to be $\mu_{\text{region}(i)} \sim N(0, \sigma^2_{\text{region}})$. The baseline and movement-related risks were obtained by conversion of the corresponding model intercepts and movement-associated coefficients as:

$$\text{Baseline risk} = \frac{exp(\text{intercept})}{1 + exp(\text{intercept})} \quad ;$$

$$\text{Movement-related risk} = \frac{exp(\text{intercept} + \log \text{odds ratio [movement]})}{1 + exp(\text{intercept} + \log \text{odds ratio [movement]})} - \text{Baseline risk}$$

where the movement-related risk is an attributable risk with a range of -1 to 1 (negative values denoting a protective exposure). Considering the pattern of the annual baseline risks hand in hand with the finding of a higher risk of infection after 2005 (Mweu et al., 2012a), we assessed whether this pattern corresponded to two distinctive risk profiles operating before and after 2005. This was originally undertaken by merging the year-specific data into a repeated measures dataset, and thereafter including the variables time (as binary) and herd (nested within region) as fixed and random effects respectively, in a mixed-effects logistic regression model.

To examine for the presence of any second-order (small-scale) spatial effects in the data, isotropic semivariograms of standardised Pearson residuals obtained from the year-specific models were plotted (Pfeiffer et al., 2008). Second-order effects result from the spatial correlation of a process and as such describe

the tendency for deviation in values of the process from its mean to follow each other in neighbouring sites (Ripley, 1981). Accordingly, this analysis facilitated the investigation as to whether geographically close herds were more similar than those geographically distant and the extent to which this occurred. The 95% simulation envelopes of the semivariograms were based on 499 Monte Carlo permutations and were produced in R software using the *geoR* package (Paulo et al., 2001). Additionally, anisotropic semivariograms at angles 0, 45, 90 and 135 degrees (with tolerance of 22.5 degrees) were graphed to assess whether any detectable spatial dependency varied with direction.

2.2.4 Predicted risk surfaces

Fitted risk values for the locations of all herds extracted from the year-specific mixed-effects logistic regression models were converted into continuous risk surfaces specific for each year by applying ordinary kriging. Kriging is an interpolation technique that predicts unknown values from data observed at known locations based on weights typically modelled by a semivariogram function (Bailey and Gatrell, 1995). The kriging weights in the present study were based on exponential models fitted to empirical semivariograms of the input data. As kriging holds a merit in allowing the errors of the imputed values to be estimated (Haining, 2003), standard error maps associated with the kriged surfaces were also produced. The analysis was carried out using the Geostatistical Analyst Extension in ArcGIS 10.1.

3. Results

An increasing trend in the pooled herd-level incidence risk of *S. agalactiae* was observed over the 10-year period, with the highest risk recorded in 2005 (Table 1). With the exception of the year 2000, the proportion of herds with at least one animal imported was greater than 50%. The highest proportion was noted in 2005 at 60.16%. The temporal distribution of the study periods was uneven (Fig. 1); the median times between screenings being longest in 2001 and 2005 (Table 1). As indicated by the study period interquartile ranges in Table 1, the middle 50% of the herds were screened over longer time spans between 2000 and 2005 compared to the period extending from 2006 to 2009.

The actual risk of a herd becoming infected with *S. agalactiae* varied spatiotemporally (Fig. 2). For the period between 2000 and 2004, the risk was lower and more homogeneously distributed compared to the period spanning 2005 to 2009. In this latter period, foci of highest risk (0.21 – 1) were noted in eastern Zealand, with other relatively smaller pockets observable in northern Funen and the western part of Zealand (see area references in the top left map of Fig. 2). However, owing to the low density of dairy herds in Zealand, the uncertainty around the computed kernel density risk estimates for this region is expected to be high.

After accounting for spatial heterogeneity in the underlying population at risk in each year, the year-specific observed difference *K*-functions showed no evidence for spatial clustering of cases relative to the population of herds at risk in each year (Fig. 3).

The annual movement-related incidence risks revealed a non-significant pattern ($P>0.05$) over the 10-year span (Fig. 4). Contrastingly, the annual baseline incidence risks demonstrated two characteristic patterns ($P<0.001$) before and after 2005; the risk being higher for the latter period. Notably, the annual baseline risks

were higher than the movement-related risks. The year-specific semivariograms of the model residuals demonstrated no evidence for the existence of significant spatial dependency (Fig. 5) i.e. geographically close herds were no different with respect to their status compared to those distant. Moreover, the anisotropic semivariograms established that the spatial distribution of the residuals did not vary with direction. As was the case with the actual risks, the predicted risk of a herd becoming infected with *S. agalactiae* varied spatiotemporally (Fig. 6). Two patterns of risk were distinguishable in the period before and after 2005. For the former period, the risk was rather inhomogeneously distributed (though lower than the latter phase), with herds located in certain areas of Jutland (and partly Funen) being at greater risk. In the latter period, the probability of a herd contracting *S. agalactiae* was reasonably uniform throughout the country. The range of the standard error values associated with the predicted risk values was 0.0003 to 0.03. The error values were highest in 2008 and 2009 owing to the comparatively few numbers of herds in those years (Fig. 7).

Table 1

Annual pooled incidence risks, proportions of open herds and median study periods as well as the annual baseline and movement-related incidence risks derived from year-specific mixed-effects logistic regression models during the period 2000 – 2009.

Year	Incidence	Population at risk	[a]Pooled incidence risk	[a, b]Proportion of open herds	[c]Median study period (interquartile range)	[a]Baseline incidence risk (95% CI)	[a]Movement-related incidence risk (95% CI)
2000	65	9138	0.71	46.34	11.60 (2.70)	0.88 (0.65 – 1.19)	-0.33 (-0.41 – 0.05)
2001	78	8076	0.97	52.17	18.47 (5.10)	0.50 (0.24 – 1.02)	-0.02 (-0.10 – 0.49)
2002	73	7068	1.03	50.42	11.20 (1.63)	0.87 (0.56 – 1.34)	0.05 (-0.19 – 0.90)
2003	87	6924	1.26	59.06	11.00 (6.77)	0.86 (0.55 – 1.36)	0.60 (0.04 – 2.26)
2004	56	6416	0.87	57.90	10.83 (10.23)	0.69 (0.44 – 1.08)	0.28 (-0.08 – 1.54)
2005	127	5246	2.42	60.16	15.20 (7.40)	1.70 (1.00 – 2.89)	0.47 (-0.12 – 2.36)
2006	80	4873	1.64	55.30	9.30 (0.67)	2.27 (1.28 – 3.99)	0.14 (-0.41 – 2.47)
2007	96	4495	2.14	55.88	13.10 (0.97)	2.07 (1.48 – 2.89)	0.65 (-0.19 – 2.75)
2008	101	4201	2.40	56.82	12.47 (0.53)	2.80 (2.07 – 3.77)	0.04 (-0.66 – 1.85)
2009	91	4015	2.27	54.35	12.50 (0.50)	1.72 (1.06 – 2.78)	0.95 (0.01 – 3.74)

[a]Expressed as a percentage.

[b]Proportion of herds with at least one animal moved in.

[c]Expressed in months.

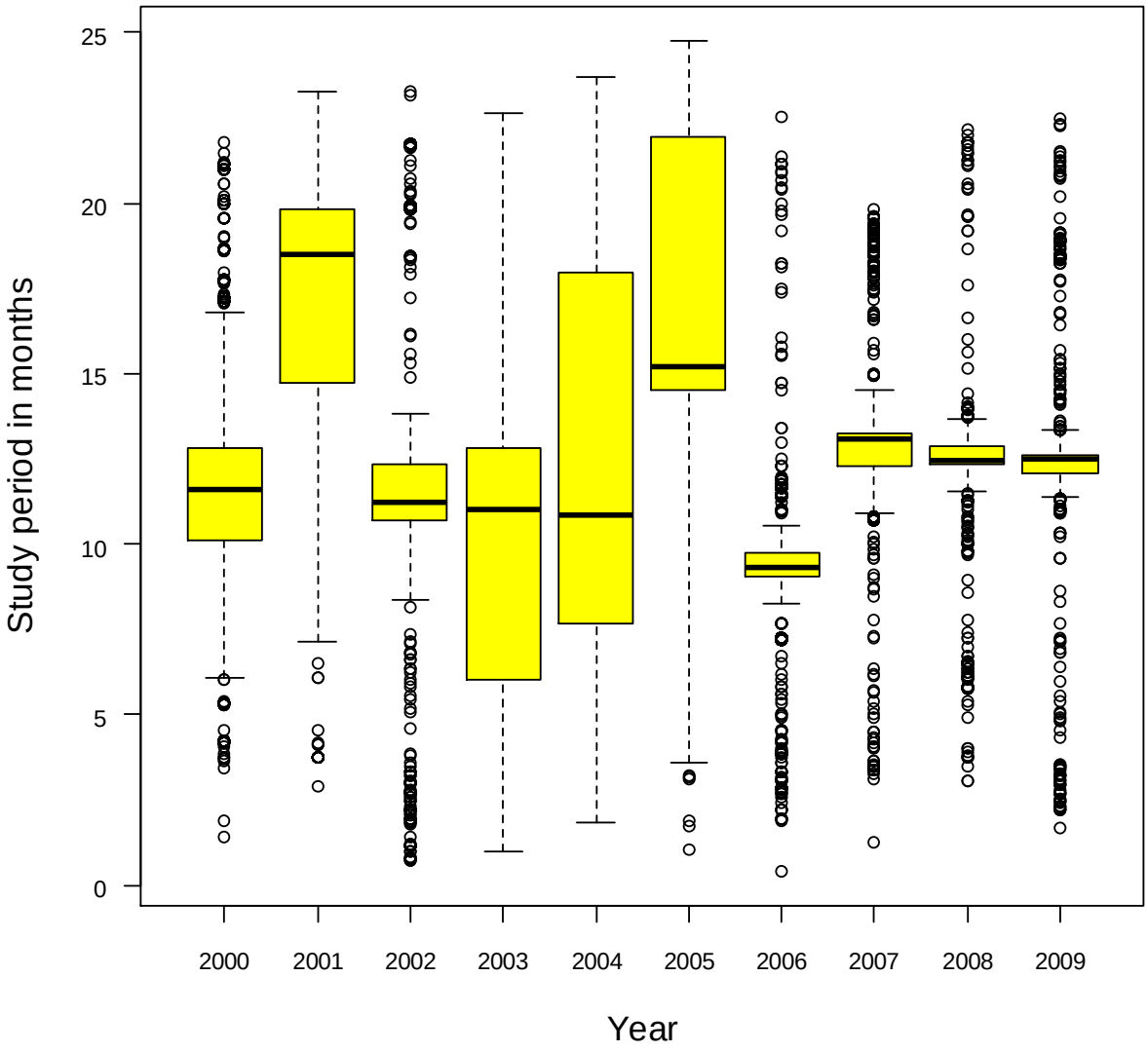

Fig. 1. Plot of the distributions of the year-specific times between screenings for *Streptococcus agalactiae* in Danish dairy herds during the period 2000 – 2009.

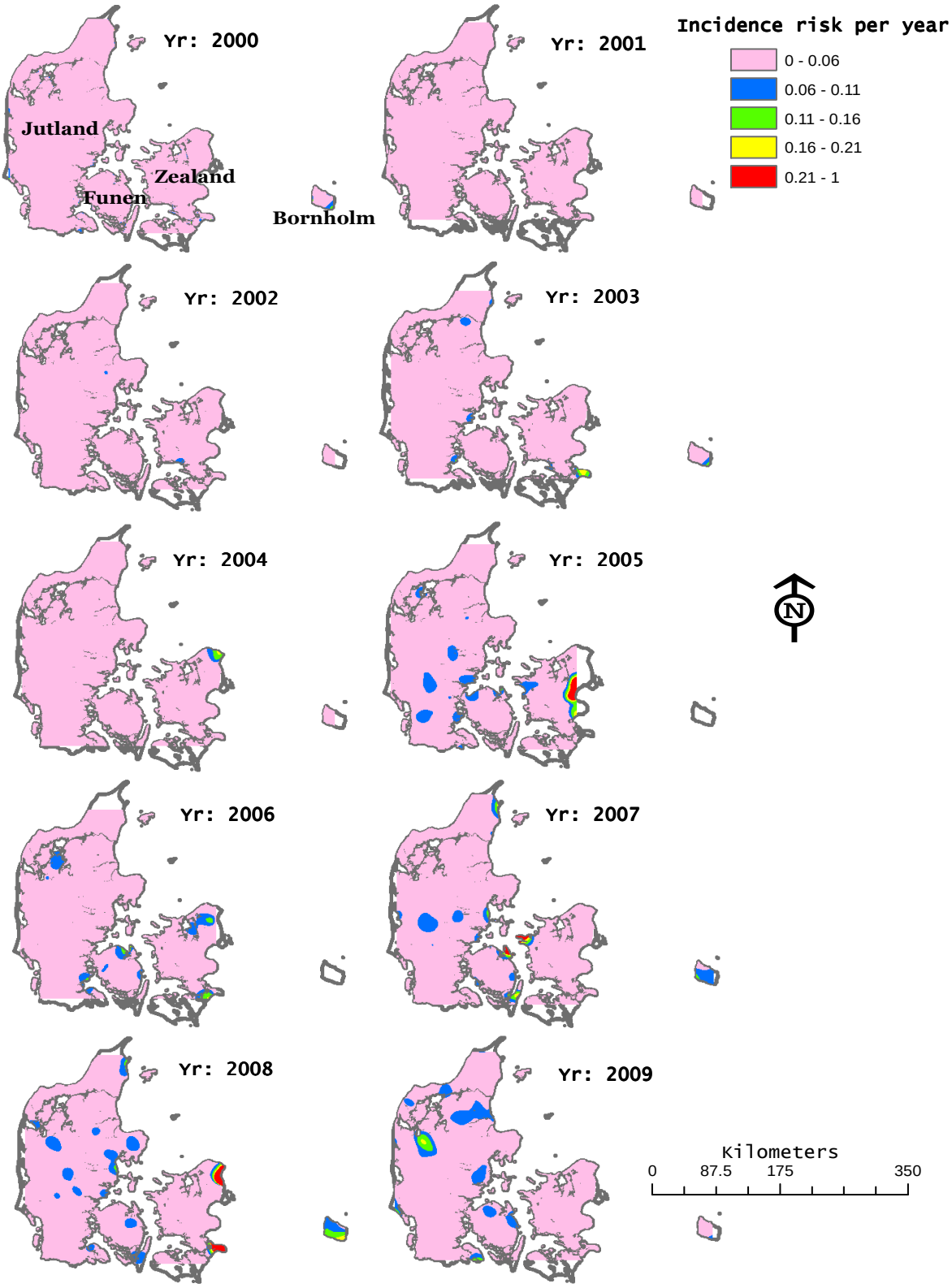

Fig. 2. Kernel density risk surfaces displaying the actual risk of a herd becoming infected with *Streptococcus agalactiae* in a given year during the period 2000 – 2009.

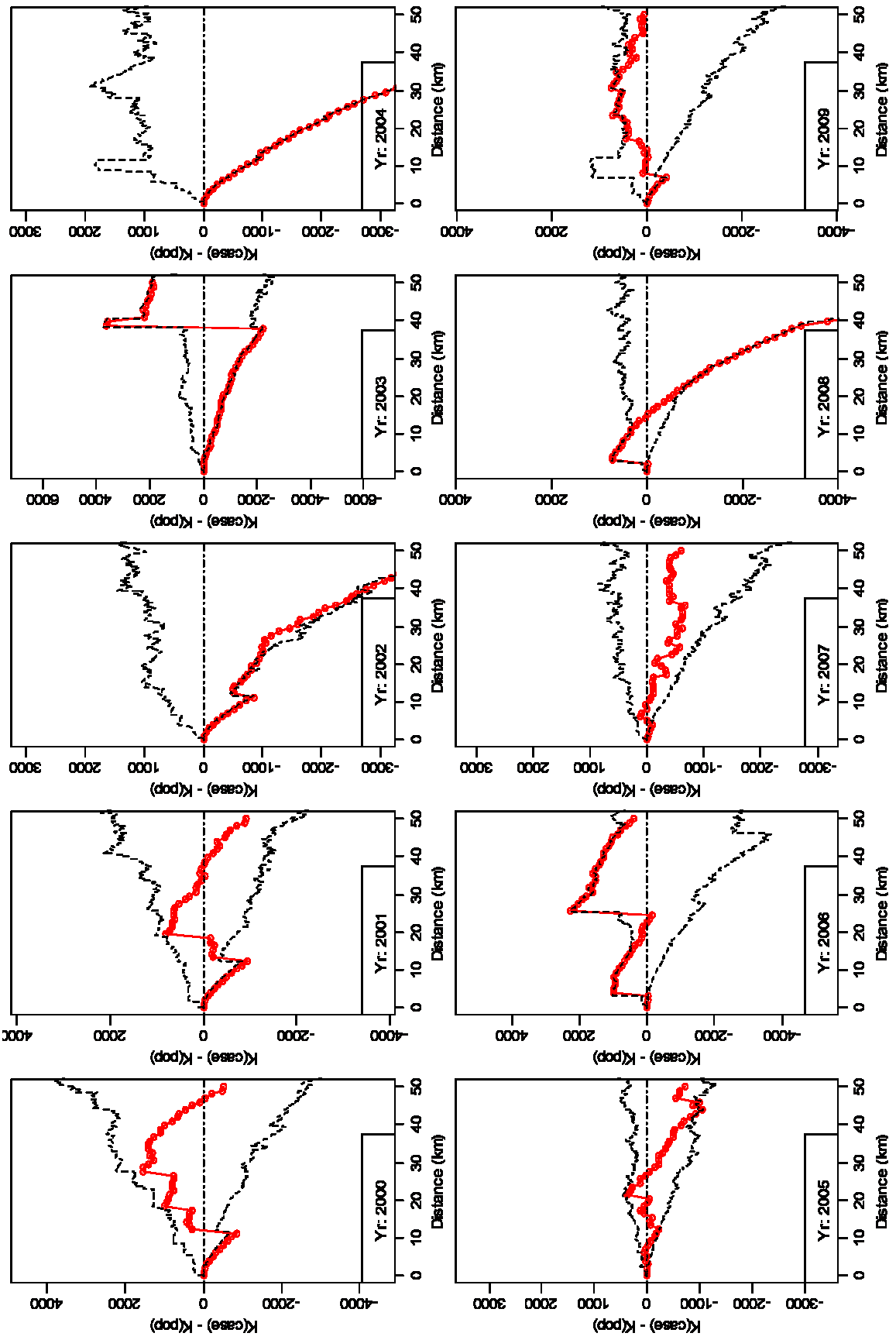

Fig. 3. Observed difference *K*-functions (with 95% simulation envelopes denoted by black dashed lines) for cases and the population of Danish dairy herds during the period 2000 – 2009.

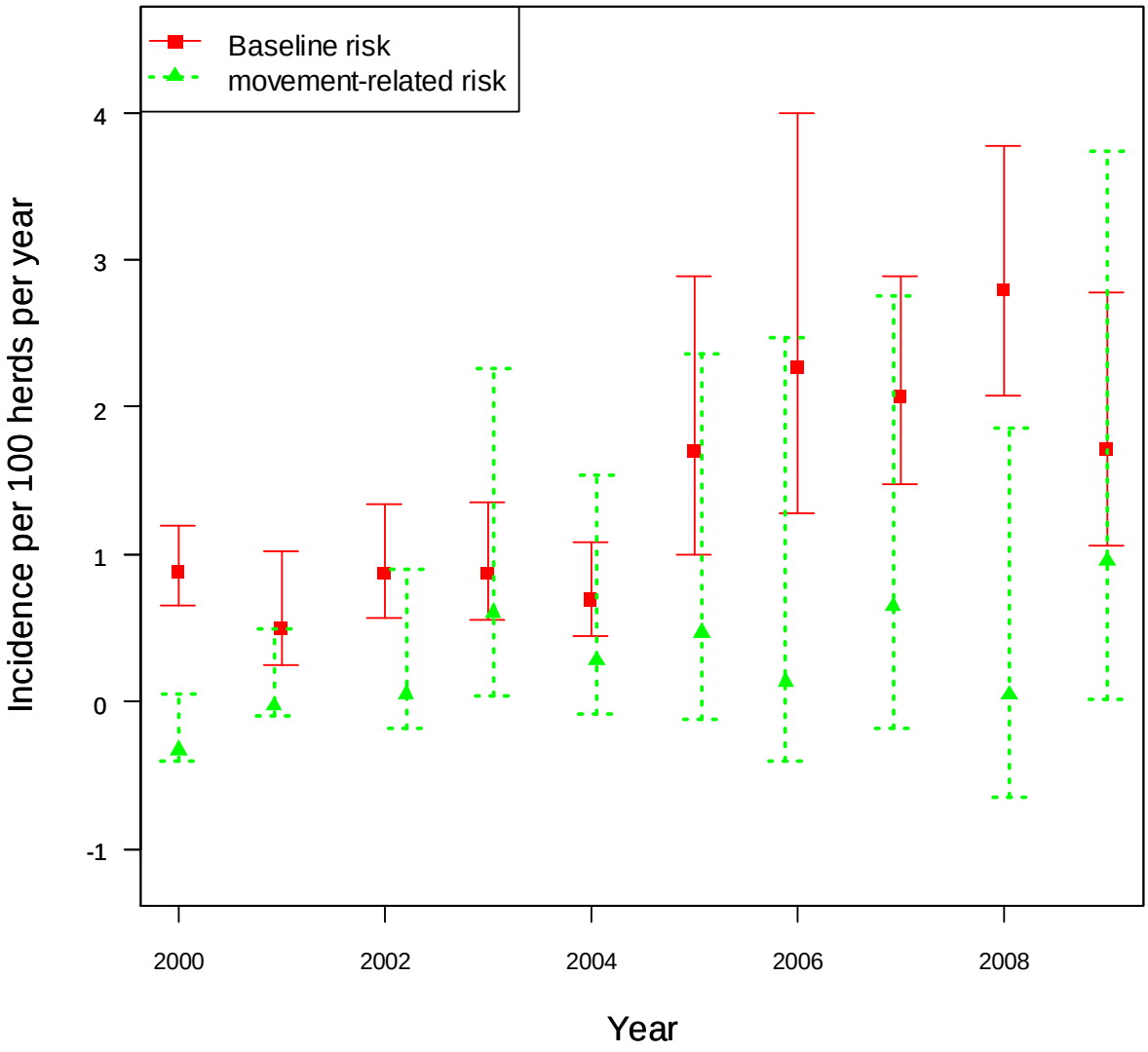

Fig. 4. Plot of the annual baseline and movement-related incidence risks of *Streptococcus agalactiae* in Danish dairy herds during the period 2000 – 2009.

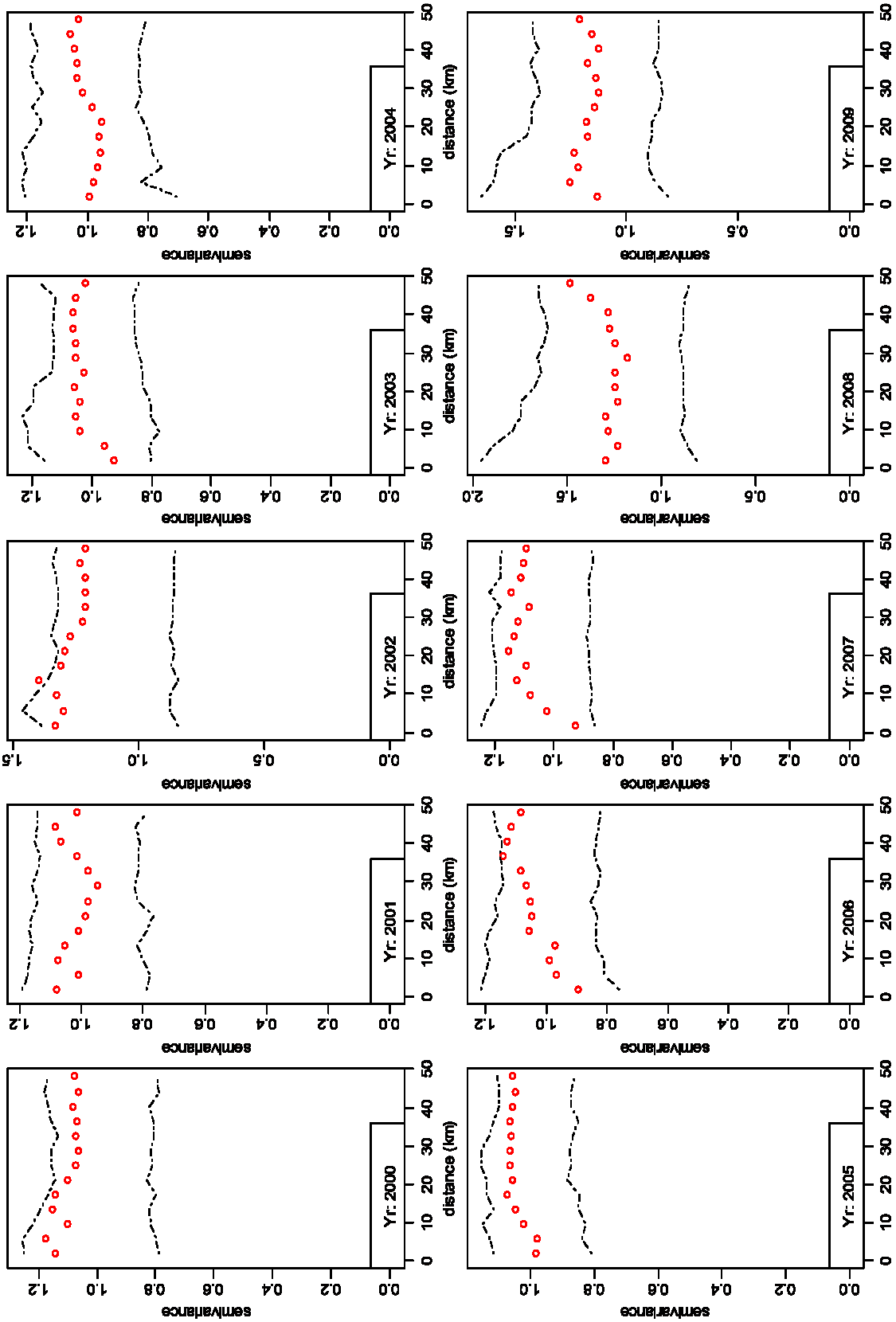

Fig. 5. Empirical semivariograms (with 95% simulation envelopes denoted by black dot-dashed lines) of the residuals of the year-specific mixed-effects logistic regression models during the period 2000 – 2009.

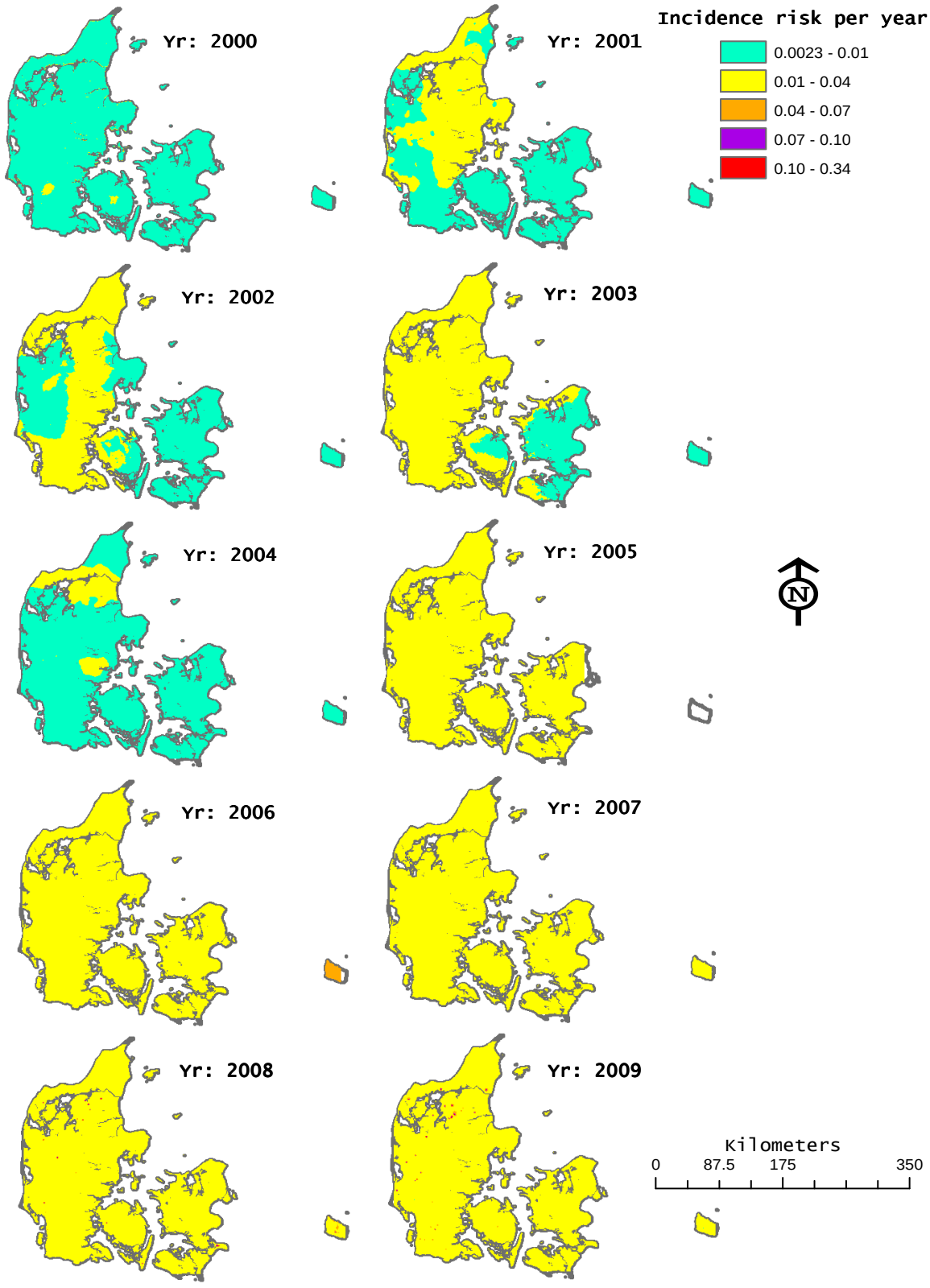

Fig. 6. Kriged risk surfaces displaying the predicted risk of a herd becoming infected with *Streptococcus agalactiae* in a given year during the period 2000 – 2009.

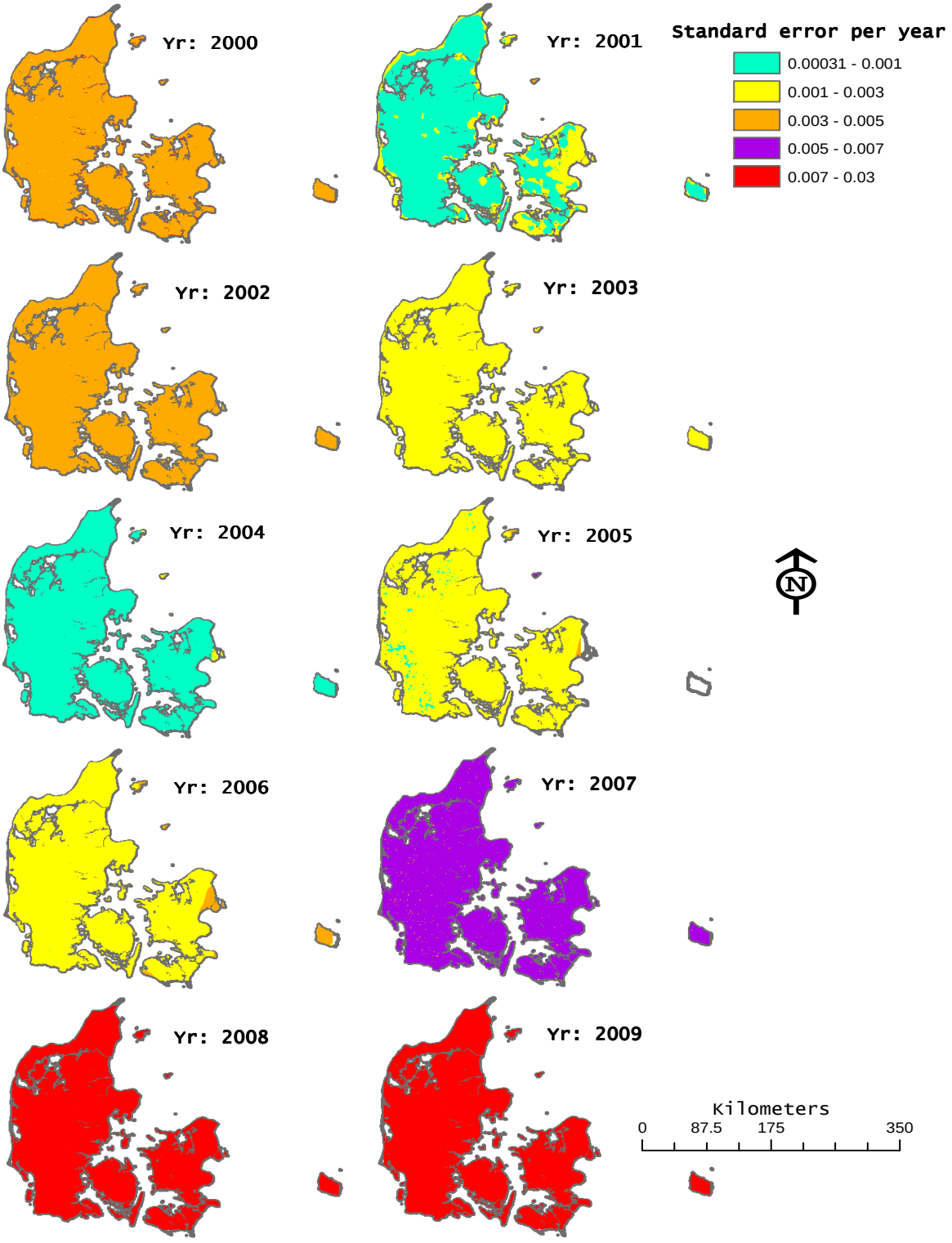

Fig. 7. Standard error maps associated with the predicted risk of a herd becoming infected with *Streptococcus agalactiae* in a given year during the period 2000 – 2009 (as displayed in Fig. 6).

4. Discussion

Analysis of the longitudinal data has demonstrated a spatiotemporal evolution of the predicted risk of infection with *S. agalactiae*; the risk of a herd becoming infected being more homogeneous and higher during the period following 2005. In the same vein, the annual baseline incidence risks exhibited two distinctive patterns before and after 2005; the latter phase corresponding to the greater risk. However, the analysis neither showed evidence for both spatial clustering and dependency between herds nor any significant role played by acquisition of animals in inducing new infections with the pathogen. The absence of spatial aggregation of infected herds during the 10-year period suggests that after adjusting for the effects of known covariates (in this case region and animal introductions) on the risk of infection, the source of the remaining risk in the year-specific models may reside in within-herd characteristics as opposed to spatially-varying factors such as sharing of farm equipment, veterinarians, inseminators, relief milkers, or local trade in animals (Benschop et al., 2006; Pfeiffer et al., 2008). This within-herd risk is exemplified by the pattern of the annual baseline risks observed in this study whose key source is largely ascribed to the within-herd human factor (Jensen, 1985; Zadoks et al., 2011). Conceivably, farmers and within-herd based relief milkers share a sizeable part of the blame in view of the amount of contact time spent with the milking herd. In one Danish study, ribotyping of isolates derived from dairy workers and bovine milk samples revealed ribotype similarities suggesting the existence of a common origin (Jensen and Aarestrup, 1996) whereas in a separate one, 5% (4/77) of interviewed owners of infected herds reported having undergone medical treatment (Katholm, 2010). It is however noteworthy that in both studies, a temporal sequence of the infection events was not established. Considering the commonness of the human element within the population of herds, it would be generally expected that the probability of herds acquiring *S. agalactiae* infection would be rather uniformly distributed in space as was witnessed after 2005 in the present study. Therefore, any spatial variation in risk could be presumed to be correlated to the degree of exposure to within-herd personnel, which indirectly relates to the level of biosecurity (Villaroel et al., 2007). In Denmark, the number of dairy herds has been on the decline from 9886 to 4258 in the course of the period 2000 to 2009 (Mweu et al., 2012a), although accompanied by gradually increasing herd sizes. This growth pattern may heighten the demands for human labour bringing about temporal variations in risk.

The characteristic change observed in 2005 coincides with a repeal in the *S. agalactiae* control policy effected in the same year that saw the earlier ban on movements from *S. agalactiae* infected herds lifted paving way for trade from the affected herds (Anon., 2005). Although this shift in policy is evidently supported by the finding that new receipts of animals played no significant role in determining incidence, a presumable consequence of this move could well have been the implied perception by farmers that *S. agalactiae* was under control prompting their adoption of a more relaxed approach to biosecurity that would catalyse the 2005 surge in baseline incidence. Such opportunities for 'lowering the guard' may be readily seized especially when farmers fail to grasp the value of maintaining costly preventive strategies (Hujips et al., 2008). The finding that the movement-related risks remained stable following the reversal of the movement ban may connote that, relative to the human strains of *S. agalactiae*, bovine strains are supposedly less resistant and thus more amenable to existing management measures. As a case in point, Dogan et al. (2005) carried out a comparative study on the phenotypic and genotypic characteristics of 52 human and 83 bovine *S. agalactiae* isolates and demonstrated that resistance to tetracycline and erythromycin was more common among human (84.6% and

26.9%, respectively) than bovine (14.5% and 3.6%, respectively) isolates. If indeed the occurrence of *S. agalactiae* in predominantly naïve herds is as a result of spill over of human *S. agalactiae*, Zadoks et al. (2011) contend that eradication of the pathogen may be infeasible. Nonetheless, this work signifies the need for bolstering biosecurity measures.

In a bid to minimise the risk of introducing *S. agalactiae* into susceptible herds, the pressing call for strengthening within-and between-herd biosecurity measures cannot be overemphasised. With regards to within-herd biosecurity, granted the potential risk of infection transmission from humans to cows, it is advisable for owners of herds in high risk regions to consider reducing the number of relief milkers and barring external personnel from handling cows in parlours (Barkema et al., 2009). As supplementary measures, education of milking personnel on personal hygiene and mastitis prevention, together with provision of gloves and hand-washing facilities should be prioritised (Villaroel et al., 2007). In fact, gloving has been shown to reduce the bacterial load on milkers' hands by 75%, and if the gloves are disinfected prior to being worn, the load decreases by 98% (Olde Riekerink et al., 2008). Despite the insignificant threat of importing *S. agalactiae* from new animal acquisitions realised from the current study, it would still be preferable that herds remain closed to ensure between-herd biosecurity. However, owing to superseding interests in fulfilling herd genetic improvements and expansion goals, purchase of animals may be necessary. In such cases, it has been recommended that both the history of the herd of origin and the animal to be purchased be established (Keefe, 2012). In Denmark, farmers can readily retrieve this information from a public database (the *B*-register) that stores data on all *S. agalactiae* culture-positive herds (Katholm et al., 2012). Barkema et al. (2009) offer useful guidelines that could facilitate the acquisition process: importantly, (1) the herd of origin should have a geometric mean BTM somatic cell count of less than 200,000 cells/mL for at least one year and it should not have tested positive for *S. agalactiae* in the last two years and (2) prepartum heifers without udder, teats and milk abnormalities should provide optimal candidates.

There are a couple of limitations inherent to the present study. The presence of *S. agalactiae* in the BTM is often construed as a direct reflection of infected udder quarters in a typical herd (Keefe, 1997). However, potential cross-contamination arising either during milk collection associated with residual milk from previously sampled infected herds or during processing at laboratories is possible (Andersen et al., 2003). As this study is not immune to the effects of potential cross-contamination, it is plausible that some herds were positively misclassified and consequently, the computed incidence risks would be overestimates of the actual risks. Bacteriological culture of BTM has been shown to have an estimated sensitivity and specificity of 68.0% and 99.7%, respectively (Mweu et al., 2012b). Since the spatial algorithms employed in the study could not permit incorporation of the test's characteristics, the resulting estimates are liable to non-differential misclassification. As such, the estimates should only be viewed as apparent. An obvious drawback afforded by the use of interval-censored data is the inability to determine the exact time that events occur, which therefore impedes the estimation of relevant risk periods (Dohoo et al., 2009). In light of this, the computed study periods should be viewed as proxies for the corresponding risk periods. Edge effects are seen as challenges to spatial analysis. They arise as a result of data locations at the periphery of a study area having fewer neighbours than those at the centre of the study area (Pfeiffer et al., 2008). Considering the robustness of the

available data, the main patterns identified in the study are unlikely to have been substantially influenced by potential edge effects.

As an adjunct to the present study, a prospective research area that could prove promising with the advent of molecular sequence data is the investigation of *S. agalactiae* strain-specific transmission dynamics. This could provide a useful understanding of their transmission potentials and in turn the determination of their threshold levels for control. Furthermore, this work paves way for risk factor studies that could elucidate potential sources of within-herd risk.

5. Conclusion

Analysis of the data spanning the period 2000 to 2009 has demonstrated that the predicted risk of a herd becoming infected with *S. agalactiae* varied spatiotemporally; the risk being more homogeneous and higher in the period after 2005. Moreover, the annual baseline incidence risks indicated significant but distinctive patterns before and after 2005, where the risk of infection was higher in the latter phase. Contrastingly, the annual movement-related risks revealed a non-significant pattern over the 10-year period. There was neither evidence for spatial clustering of cases relative to the population of herds at risk nor spatial dependency between herds. Notwithstanding this, there is need to step up within-herd biosecurity to minimise the risk of introducing *S. agalactiae* into naïve herds.

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CHAPTER 6:

GENERAL DISCUSSION, CONCLUSIONS AND FUTURE PERSPECTIVES

6.1 GENERAL DISCUSSION

6.1.1 General concerns

In practice, diagnostic tests can lend themselves to application at both the individual and population-level. At the individual level, the goal for their use is often to identify individuals with the disease of interest for effective control (Connell and Koepsell, 1985). At the population level however, their usage is fairly wide and is unrestricted to prevalence and incidence estimation, quantitative risk assessment, risk-factor studies, disease mapping and infectious disease modelling (Greiner and Gardner, 2000a). Nevertheless, it is noteworthy that the accuracy of the tests used determines the quality of the resulting data that are available for research purposes, such that failure to make appropriate adjustments for the Se and Sp of the tests in analyses inevitably gives rise to biased estimates that can lead to flawed conclusions (Greiner and Gardner, 2000a). For instance, since the magnitude of the incidence and prevalence of infections is reflective of the degree of success of existing control measures, misclassification of their estimates is bound to under or overemphasise the performance of control efforts. With this in mind, except in chapter 3b where the apparent (test) estimates of the herd-level incidence and prevalence of *S. agalactiae* infection are corrected for the accuracies of bacteriological culture and the PathoProof Mastitis PCR, interpretation of the results in this thesis should be made with caution.

In longitudinal studies of infections with contagious pathogens, it is preferable to have data collected at intervals shorter than the average risk period for the infection in question (Dohoo et al., 2009). Availability of such data could enable the capturing of seasonal fluctuations in the frequency of infections particularly typified by recurrent infection-recovery events. This in turn could assist responsible authorities to target control efforts during seasons when the risk of infection is highest. Spontaneous clearance of infections with human strains of *S. agalactiae* has been observed (Jensen, 1982), rendering infections with the pathogen suitable for the aforementioned assessment (Andersen et al., 2003). However, because only annual data were available, this evaluation was not possible.

Cross-contamination of BTM samples can occur either during milk collection as a result of transfer of residual milk from previously sampled infected herds or processing of samples at the laboratory (Andersen et al., 2003). Andersen et al. (2003) observed that of the 100 dairy herds enrolled in a study to evaluate the Danish *S. agalactiae* surveillance system, the pathogen was isolated from BTM samples in 6 out of 10 truck routes serving 60 herds signifying a non-negligible role of milk trucks in between-sample contamination. Therefore, in light of this finding, false-positive misclassification of herds is likely, which could bias the estimates given in this thesis.

6.1.2 Further clarifications and new perspectives

The purpose of this thesis was to examine the epidemiology of *S. agalactiae* infections in the national Danish dairy herd population with the goal of understanding the accuracy of the available diagnostic tests, frequency and transmissibility of the pathogen, the contact pattern of the cattle herd population, the geographical and temporal patterns of infection. Knowledge of these characteristics will be pivotal to supporting future *S. agalactiae* control decisions.

6.1.2.1 Performance of bacteriological culture and PathoProof Mastitis PCR

In chapter 2, using a Bayesian framework, the Se and Sp of the BTM bacteriological culture and the PathoProof Mastitis PCR assay were inferred. At a Ct cut-off value of 40, the PCR assay had the highest combined accuracy rendering the cut-off a suitable threshold below which BTM samples should be scored positive for *S. agalactiae*. At this cut-off, the Se of PCR was higher (95.2%; 95% PCI [88.2% – 99.8%]) than that of culture (68.0%; 95% PCI [55.1% – 90.0%]), although the Sp of culture was higher (99.7%; 95% PCI [99.3% – 100.0%]) compared to that of PCR (98.8%; 95% PCI [97.2% – 99.9%]).

Despite culture being qualitative nature, its evaluation against the quantitative PCR afforded an opportunity to assess its performance at varying levels of CFU in the BTM (target condition). The analysis revealed that the accuracies of the tests depended on the target condition under consideration. In particular, at the lowest Ct value of 31, the target condition was considered to feature primarily herds with high levels of infection (high CFU levels), of which culture was superior at detecting – though at the same cut-off the PCR yielded fewer false positives. As the cut-off was further raised, an increasing number of herds with reducing concentrations of bacteria in their BTM were added to the existing pool constituting the target condition, such that at the highest Ct value of 40 the entire spectrum of infection was represented though dominated by herds with low levels of infection that PCR was better at diagnosing. Essentially, the Se of culture diminished while that of PCR increased as more herds with decreasing BTM CFU levels were included in the case definition. The reverse was true with regards to Sp.

There are key implications of this phenomenon. Cows infected with human strains of *S. agalactiae* have been shown to excrete lower levels of bacteria per mL of milk than those infected with bovine strains (Jensen, 1982). Given the predominance of human strains in Danish dairy herds (Zadoks et al., 2011), bacteria concentrations in the BTM of a majority of herds are likely to be low, thus justifying the requirement of the more sensitive PCR assay for their detection. However, as the between-herd prevalence of *S. agalactiae* declines, given the comparably lower Sp of the PCR, the predictive value of its positive result (PPV) is bound to be increasingly lower than that of culture (undermining the confidence in its positive result), therefore warranting confirmation by the more specific culture (Table 1). From the table, it is also apparent that despite PCR having a higher Se, considering the low prevalence of the infection, the negative predictive values (NPV) of the tests remain reasonably comparable, with further decreases in prevalence being accompanied by increasing NPV of culture. At lower prevalences, a serial combination of the test results should assure complete confidence in a positive result without significantly compromising faith in a negative outcome.

Table 1

Population-specific prevalences, positive and negative predictive values of bulk tank culture and PCR tests as well as predictive values of the tests' serial interpretation at PCR cut-off <40

Population	Prevalence (95% PCI)	Test				Serial interpretation	
		Culture		PCR			
		PPV (95% PCI)	NPV (95% PCI)	PPV (95% PCI)	NPV (95% PCI)	PPV (95% PCI)	NPV (95% PCI)
1	4.3 (2.4; 6.6)	90.0 (77.9; 99.3)	98.5 (97.2; 99.7)	78.9 (50.7; 98.9)	99.8 (99.3; 100.0)	99.9 (99.5; 100.0)	98.4 (96.9; 99.6)
2	6.3 (4.2; 8.3)	93.2 (85.6; 99.5)	97.8 (96.4; 99.5)	84.5 (61.1; 99.3)	99.7 (99.1; 100.0)	99.9 (99.7; 100.0)	97.6 (96.0; 99.4)
3	6.4 (4.3; 8.5)	93.3 (86.1; 99.5)	97.8 (96.3; 99.5)	84.8 (61.7; 99.3)	99.7 (99.1; 100.0)	99.9 (99.7; 100.0)	97.6 (95.9; 99.4)
4	8.4 (5.9; 10.9)	94.9 (89.3; 99.6)	97.1 (95.2; 99.4)	88.1 (69.0; 99.4)	99.5 (98.8; 100.0)	99.9 (99.8; 100.0)	96.8 (94.8; 99.1)

Serial testing: Se, 64.7 (51.5; 86.4)
Sp, 100.0 (100.0; 100.0)

As the PCR targets DNA, it is likely that a number of herds with Ct values close to the cut-off of 40 may simply be reactors devoid of an active infection. In such cases, besides bacteriological confirmation of the positive results, the herd's clinical history along with its bulk tank milk somatic cell count (BTMSCC) should be taken into consideration (Schukken et al., 2010). Furthermore, screening of individual cows in PCR-positive herds could serve to confirm the herds' infection statuses. Apart from differences in their detection techniques (agent isolation for culture versus DNA detection for PCR), dissimilarities in their respective inoculation volumes may explain the differences in their Se estimates; 120 µL and 350 µL milk inocula were used for culture and PCR respectively. Godkin and Leslie (1993) suggest that increasing the volume of milk cultured may augment the Se of culture. Although, on the one hand, the PCR demonstrates suitability for use in low-*S. agalactiae* prevalence settings such as Denmark, on the other hand, bacteriological culture may be well suited for high prevalence areas e.g. Latin America (Elias et al., 2012; Ceballos, 2013) where within-herd prevalences of *S. agalactiae* and hence BTM CFU levels are expected to be high.

To assess whether the accuracies of the tests were affected by the herd-level covariate effects of milking system, production type or herd size, stratification of the four populations of herds by the levels of each covariate was attempted. Stratum-specific estimates of the Se and Sp of both tests were then computed. The reason for carrying out the stratified analysis was based on the observation that culture Se varied depending on the within-herd prevalence of *S. agalactiae* (Gonzales et al., 1986). As this prevalence is also expected to differ between large and small herds, organic and conventional ones as well as herds with and without automatic milking systems, the performance of the tests across the levels of the covariates is anticipated to be dissimilar. Nevertheless, no significant covariate effects were realised by the analysis. The failure to demonstrate significant effects could have been occasioned by the simple stratified analysis undertaken – corresponding to univariable analysis – which could have resulted in residual confounding (Coughlin et al., 1992). Alternatively, a logistic regression approach could have been employed where the test outcome would

be modelled as a function of the true infection status and the given set of covariates presumed to affect the Se and Sp of the tests (Greiner and Gardner, 2000b). Nevertheless, as none of the covariate-specific Bayesian posterior probabilities (POPR) (frequentist *P*-value analogue) met a defined criterion (POPR<0.20) for inclusion in a multivariable model (chapter 2, Table 2), the stratified approach seemed to suffice. Furthermore, demonstrating a significant effect in more than one covariate could hamper the practical usefulness of the tests.

Two particular assumptions underpinning latent class analysis relate to constancy of the Se and Sp of the tests and differences in the prevalences of the populations studied (Hui and Walter, 1980). Despite being unstated in chapter 2, constancy of the tests' estimates was evaluated by carrying out a stepwise exclusion of the individual populations followed by inspection of the resultant estimates. There were no substantial changes observed and therefore the assumption remained valid. Pertaining to the assumption about differences in population prevalences, Toft et al. (2005) showed that larger differences in the prevalences increased the precision of the estimates. The minimal differences in the prevalences observed in our study were reflected on the fairly wide credibility intervals of the Se estimates (chapter 2, Table 3). However, granted a large pool of non-infected herds, the precision of the Sp estimates was high.

6.1.2.2 Frequency and transmission characteristics of *S. agalactiae*

In chapter 3a, the annual herd-level apparent incidence rates and prevalences as well as rates of the demographic (entry and exit) parameters, recovery and transmission of *S. agalactiae* infection during the period 2000 to 2009 were derived. In chapter 3b, the test-based estimates of incidence and prevalence were corrected for diagnostic misclassification and the analysis further extended to cover the periods from 1966 to 1999 and 2010 to 2011. It was demonstrated that, during the culture-based phase of the surveillance programme between 1966 and 2008, the apparent prevalences (Ap) underestimated the true prevalences (Tp) whereas the apparent incidence risks (A_i) overestimated the corresponding true incidence risks (T_i). The finding of the Ap being lower than the Tp pointed to the inadequacy of single bacteriological cultures of BTM in the detection of existing herd infections, affirming the necessity of repeated testing with parallel interpretation of test results to improve the Se of culture. Given the result of the A_i being higher than the T_i, culture nevertheless demonstrated sufficiency in identifying new herd infections. Although being capable of detecting new infections for prompt control, its inadequacy in identifying existing infections was expected to have contributed to the silent persistence of infections particularly after 1995 when screening of samples became annual (Andersen et al., 2003). Prior to 1995, the undertaking of regular repeated surveys could have played a central role in enhancing the rapid detection of infections for control. Although it is likely that during the infancy stages of the surveillance programme (when within-herd prevalences were probably high), the Se of culture could have been sufficiently high to facilitate control efforts. Despite cattle purchases being shown to increase the risk of new herd infections (Agger et al., 1994), the imposition of the movement ban seemed to be effective at maintaining the stability of incidence during the period between 1992 and 2004 (chapter 3b, Fig. 1). It could be speculated that considering the importance of cattle movements in spurring new infections during this period coupled with small average herd sizes and thus low human labour demands, a predominance of bovine over human strains was likely, to which the ban successfully targeted.

The increase in *S. agalactiae* infection frequency from 2000 to 2009 indicated a re-emergence of the pathogen in the Danish dairy herd population. Several explanations for the resurgence could be postulated:

- With increasing herd sizes and hence a growing demand for human labour, contacts with the milking herd may intensify resulting in a preponderance of human over bovine strains (Zadoks et al., 2011). This hypothesis that seemingly leans more towards a spillover of human strains into the bovine population as opposed to a re-emergence of historical bovine strains has been suggested (Hakker, 2013). Granted that human strains exhibit lower susceptibility to antibiotic therapy compared to bovine strains (Dogan et al., 2005), persistence of infections may be favoured contributing to the increase in infection frequency.
- It is conceivable that with control of the infection being voluntary, over time, a waning adherence to the five-point plan for contagious mastitis pathogens in particular the use of dry cow therapy (DCT) and post-milking teat disinfection (PMTD) is expected. A 2008 survey conducted amongst Danish dairy farmers showed that only 75% and 74% of them practised PMTD and selective DCT respectively (Katholm, 2010). Since the rationale for instituting DCT and PMTD is to eliminate existing and prevent new intramammary infections (Halasa et al., 2009a, b), a sustained reduction in their uptake may lead to a rise in the frequency of *S. agalactiae* infections over time. Intensification of campaigns to minimise the use of antibiotics in milk production in Nordic countries could further exacerbate the problem (Ekman and Østerås, 2003).
- The rise in *S. agalactiae* infection frequency seems to be correlated well with an increasing adoption of automatic milking systems (AMS) amongst Danish dairy herds (Katholm and Rattenborg, 2010). A switch from conventional milking to AMS could negatively impact udder health owing to increased milking frequencies that ensure longer patency durations of teat canals, which in turn could predispose the udder to bacterial invasion (Hovinen and Pyörälä, 2011). Bennedsgaard et al. (2004) noted an increase in antibiotic treatment for mastitis in 20 Danish farms following the introduction of AMS. In a separate study, it was shown that herds milked with AMS had on average a 6.9% higher incidence risk of subclinical heifer mastitis (Santman-Berends et al., 2012).
- The reversal of the movement ban in 2005 – whose lifting was considered because the risk of transmission via animal movements was negligible – could have led farmers to trivialise the importance of controlling the infection, which would catalyse the surge in frequency after 2005.

The PCR assay demonstrated suitability for the identification of new and existing infections, with the result of its use in surveillance of BTM between 2009 and 2011 being characterised by a decline in the Tp and T_i of the infection. This finding implied that there was better promise for the elimination of *S. agalactiae* infections using PCR in the Danish dairy herd population especially in the face of declining bacterial concentrations in the BTM. The variation in the detection of existing infections by culture and PCR was argued from the viewpoint that cows in herds with existing infections could have had adequate time to mount immune responses capable of reducing the numbers of bacteria shed in the BTM to levels which could compromise their detection by culture, but could yet be sufficiently high for identification by PCR. Contrastingly, cows in newly infected herds were thought to be shedding reasonably large amounts of bacteria that could readily be detectable by both tests.

In chapter 3a, the basic reproductive ratio, R_o was estimated to be 1.10 (95% CI 1.08 – 1.22), signifying that on average each infected herd contributed to more than one new herd infection – and as expected – incidence increased. Bearing in mind that an R_o value below the unity threshold denotes that incidence will decline, the estimated value for *S. agalactiae* implies a low between-herd transmissibility for the pathogen and hence a low potential for its spread in the Danish dairy herd population. However, granted that the measure was expressed aggregately, it is probable that the transmissibility of human and bovine strains could indeed be different with bovine strains being more transmissible (Jensen, 1982). The incidence surge in 2005 was speculated to be attributable to the lifting of the movement ban in the same year that conceivably, would have resulted in a subsequent increased transmission of infection through cattle movements. Surprisingly though, the between-herd transmission (as denoted by β) remained constant before and after 2005 suggesting that the rise in incidence could likely have been driven by within-herd sources (see chapter 5).

Incidence estimates were expressed as rates in chapter 3a whereas risk estimates were computed in chapter 3b. Martin et al. (1987) contend that when rates are low (<15%), rates and risks are practically numerically similar, in which case either should suffice in incidence calculation. However in order to account for demographic changes that occur in the population over time – when averaging estimates of recovery, entry and exit – rates are preferable (Dohoo et al., 2009).

6.1.2.3 Contact structure of the population of Danish cattle herds

In the estimation of parameters in chapter 3a, it was revealed that the homogeneous-mixing assumption of dairy herds did not hold in light of overdispersion in the data implying the existence of an underlying non-random contact pattern. Homogeneity in mixing patterns approximates to a regular random network in which individuals are assumed to have constant contact with all other individuals in the population – a presumption that is biologically unrealistic (Bansal et al., 2007). We therefore sought to unravel the contact structure of the Danish cattle herd population in chapter 4 to understand its bearing on the control of contagious diseases. Even though the analysis was not specific to *S. agalactiae*, two fundamental lessons with practical relevance to *S. agalactiae* control can be drawn from the network analysis study:

- The Danish cattle herd network was found to be sparsely connected with markets being the key influential holdings mediating a disproportionate share of in and out cattle movements. Such highly connected holdings are at risk of contracting and disseminating infection to other holdings in the network, rendering the network prone to persistence of infections (Keeling and Eames, 2005). With regards to *S. agalactiae*, Agger et al. (1994) demonstrated that dairy herds that purchased cattle from markets were at an increased risk of becoming infected with *S. agalactiae*. Although a significant effect of purchases from markets was not evident in the analysis performed in chapter 5 (results not shown), it is still advisable for farmers to observe precautionary measures subsequent to acquisition of animals from markets i.e. segregation and screening of the animals prior to their introduction into the milking herd.
- The contact pattern displayed high heterogeneity where a few holdings monopolised most of the contacts while the majority only had a few contacts (scale-free nature). This is a clear departure from the random-mixing assumption in traditional epidemiological modelling and stresses the importance of accounting for the contact structure in simulation modelling of the spread and control of *S.*

agalactiae in order to prevent possible misestimation of the effectiveness of control strategies (Webb and Sauter-Louis, 2002).

Development of an exclusive dairy network had initially been considered. But because movements emanating from non-dairy premises also presented a risk for open dairy herds, a general cattle herd network was instead preferred.

6.1.2.4 Spatiotemporal patterns, baseline and movement-related risks of *S. agalactiae* infections

In chapter 5, the spatiotemporal patterns of *S. agalactiae* infection were described together with the estimation of the annual herd-level baseline and movement-related incidence risks of the infection during the period 2000 to 2009. It was shown that the predicted risk of a herd becoming infected changed spatiotemporally, with the risk being more uniform and higher after 2005. In the same breath, the annual baseline incidence risks displayed two characteristic patterns before and after 2005; the latter phase relating to the higher risk. The uniformity in the spatial distribution of risk after 2005 was considered to be attributable to within-herd characteristics that were represented by the annual baseline risks. This perspective was reached based on the following grounds:

- Absence of evidence for spatial clustering of cases relative to the underlying population of herds at risk.
- Absence of a significant role of animal introductions in the risk of new herd infections.
- Absence of evidence for the existence of spatial dependency between herds after accounting for the effects of known covariates i.e. region, animal introductions and study periods on the risk of infection ruling out the potential role of spatial risk factors such as sharing of farm equipment, veterinarians, inseminators, relief milkers or local trade in animals (Benschop et al., 2006; Pfeiffer et al., 2008).

The source of the within-herd risk was blamed on the within-herd human element (Jensen, 1985; Zadoks et al., 2011), in particular, farmers and within-herd based relief milkers who imaginably have the largest share of contact-hours per cow. However, arguably, an environmental source could likewise be postulated. Manning et al. (2010) reported recovery of the principally human ST 1 from bovine stool indicating the probability of transient bovine rectal colonisation by *S. agalactiae* whose shedding into the cow environment could serve as a potential source of infection for the udder. Interestingly, Zadoks et al. (2011) observed that ST 1 was the commonest (28%) *S. agalactiae* isolate recovered from Danish BTM samples underlining the possibility of a sizeable environmental role in the Danish dairy herd *S. agalactiae* infections. If indeed *S. agalactiae* has an environmental source, elimination of infections from the Danish population may prove infeasible. Nonetheless, to curb new environment-associated infections, the need for a hygienic, dry and comfortable environment as recommended in the expanded (to cater for environmental mastitis pathogens) mastitis control programme is vital (NMC, 2009).

Introduction of animals into dairy herds was not shown to significantly influence the risk of new herd infections. This observation not only corroborated the lifting of the movement ban, whose reversal was ascribed to a negligible risk of infection transfer via purchase (Anon., 2005a, b), but also supported the hypothesis of an insignificant contribution of bovine strains in the overall *S. agalactiae* burden in the Danish

dairy herd population. This is in stark contrast to their presumably dominant role in the early 90's (Agger et al., 1994). Another important inference that can be drawn from finding an insignificant role of animal acquisitions in driving the herd-level incidence is that animal movements may not serve as a conducive route for conveying human strains of the pathogen between herds implying a low bovine-udder adaptation of these strains. Hakker (2013) showed that, of the predominant *S. agalactiae* isolates recovered from Danish BTM samples i.e. ST 1 and ST 23 (primarily human strains) (Zadoks et al., 2011), a majority lacked a specific gene within the lac operon – believed to encode for lactose fermentation and may be essential for conferring a survival advantage in the bovine udder (Richards et al., 2011). The growing importance of human strains in *S. agalactiae* dairy cattle infections in Denmark was illustrated by the pattern of the annual herd-level baseline risks (chapter 5, Fig. 4).

At this juncture, there are two looming questions that deserve attention: (1) are *S. agalactiae* infections eliminable from the Danish dairy herd population? If so, (2) is there a justification for eliminating them? Since for years, *S. agalactiae* was regarded as an obligate pathogen whose sole reservoir was deemed to be the infected bovine udder (McDonald, 1977; Keefe, 1997), elimination of infections with the pathogen could have been foreseeable by ensuring prompt removal of the reservoir of infection through treatment and culling of chronically infected cows. However, in light of the mounting evidence suggesting potential bovine rectal colonisation and thus a credible environmental threat (Manning et al., 2010) coupled with a sizeable human reservoir (Zadoks et al., 2011), elimination of infections from Danish dairy herds may be arguably unattainable. But even if elimination were feasible, there seems to be no obvious grounds for doing so. In comparison to infections with its contagious counterpart i.e. *Staphylococcus aureus* (*S. aureus*): (1) in 2009, the herd-level prevalence of *S. agalactiae* infection in Danish dairy herds by PCR was comparably lower (7%) than that for *S. aureus* infections (91%) (Katholm et al., 2012), (2) unlike *S. agalactiae*, *S. aureus* infections tend to be chronic in nature and more refractory to treatment often leading to culling of infected cows with the need for replacement (Radostits et al., 2007). Consequently, from an economic standpoint, control of *S. agalactiae* is relatively less justifiable.

6.1.3 Recommendations

The following key recommendations can be deduced from this thesis:

- The PCR assay has been shown to have superior sensitivity to bacteriological culture and lends itself to routine use in the Danish *S. agalactiae* surveillance programme. Its adequacy in detecting both existing as well as new herd infections renders it a better choice for facilitating control efforts aimed at eliminating *S. agalactiae* infections from the population of Danish dairy herds. However, to reduce the proportion of false positives particularly when the herd-level prevalence of *S. agalactiae* infection is low, confirmation of PCR-positive herds by culture is advisable.
- There is an increasing role of human strains in the overall Danish *S. agalactiae* burden that warrants intensification of awareness campaigns amongst dairy farmers aimed at enhancing the uptake of:
  a) Within-herd biosecurity measures, in particular as pertains to close observance of hand hygiene, hand gloving and disinfection.
  b) The five-point plan for control of contagious mastitis pathogens especially with regards to PMTD and DCT.
  c) Rigorous environmental hygiene to minimise the potential risk of environment-related *S. agalactiae* infections.

6.2 CONCLUSIONS

The following conclusions can be derived based on the work described in this thesis:

- The PCR assay was shown to have higher Se but lower Sp compared to that of culture. It was shown to outperform culture in the detection of herds with low levels of infection (low CFU levels in the BTM), although culture was found to be better suited for detecting herds with high levels of infection (chapter 2).
- The PCR assay demonstrated suitability for use as a screening test for *S. agalactiae* i.e. it had both high Se and Sp. Further, it showed adequacy for use in a low *S. agalactiae*-prevalence setting such as Denmark (chapter 2).
- The tests' accuracies were unaffected by herd size, production type and milking system. Consequently, their ability to detect the infection status of herds remained the same irrespective of the level of the specific covariates under consideration (chapter 2).
- The annual herd-level apparent prevalences and incidence rates displayed increasing trends during the period 2000 – 2009, with a distinctive surge in incidence being notable in 2005. Moreover, the basic reproductive ratio was estimated at 1.1 suggesting that each infected herd would result in more than one new herd infection and thus incidence would increase. These findings implied a possible resurgence of the pathogen in the Danish dairy herd population (chapter 3a).
- Bacteriological culture was found to be inadequate in detecting existing herd infections, but was suitable for diagnosing new herd infections. By contrast, the PCR assay demonstrated sufficiency for diagnosing both new as well as existing infections. Its incorporation into the surveillance programme in 2009 was characterised by declining trends in the T_i and Tp of the infection. The test thus affords better promise for facilitating the elimination of *S. agalactiae* infections in Danish dairy herds (chapter 3b).

- The contact pattern of Danish cattle herds displayed high heterogeneity with a few holdings having a large number of contacts while the majority had few contacts (scale-free behaviour). This suggested a need to account for the heterogeneous contact structure in future simulation modelling of the spread and control of *S. agalactiae* to guard against potential misestimation of the effectiveness of control strategies (chapter 4).
- Markets were found to be the most influential holdings in the network indicating their susceptibility to infection acquisition and potential for spreading the infections. Therefore, with respect to *S. agalactiae*, purchase of cattle from markets may represent a viable risk that farmers can minimise by segregation and screening of purchases prior to their introduction into the milking herd (chapter 4).
- The risk of a herd becoming infected with *S. agalactiae* varied spatiotemporally; the risk being greater and spatially homogeneously distributed after 2005 (chapter 5).
- There was neither evidence for spatial clustering of cases relative to the underlying population of herds at risk nor spatial dependency between herds suggesting the absence of significant local spread of the infection in the population (chapter 5).
- There was no evidence for the significant role of between-herd factors in determining incidence. Instead, the analysis demonstrated that new herd infections were chiefly driven by within-herd characteristics, presumably the within-herd human factor (chapter 5).

6.3 FUTURE PERSPECTIVES

Future research work on *S. agalactiae* infections in Danish dairy herds could focus on the following:

- Within-herd risk factor studies with the prime goal of elucidating the roles of humans and the environmental component in influencing new herd infections.
- Epidemiological modelling of the effectiveness of control strategies against *S. agalactiae* with a view to establishing threshold levels for the uptake of PMTD, DCT and hand sanitation that are adequate for stabilising the frequency of infections.
- Economic analysis with the objective of assessing the impact of switching from bacteriological to PCR surveillance of BTM in the control of *S. agalactiae* infections.
- Assessment of within-herd transmission dynamics with a view to quantifying the cow-level transmissibility of human and bovine strains of *S. agalactiae*.
- Evaluation of the relationship between BTM concentration of *S. agalactiae* (CFU/mL) and PCR Ct values to determine the usefulness of BTM Ct values as proxies for the bacterial load in the BTM.

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APPENDIX:

R ANALYSES CODES

CHAPTER 2

```
#LCM model allowing for stratification

rm(list=ls())

setwd("C:/Dropbox/MARSHAL_FILES/LATENT")

library(BRugs)
library(foreign)

lcm.dat = read.dta("LCM-MOVT-XY.dta")

#Recategorize region and herd size
lcm.dat$region.cat[lcm.dat$region<=3] = 1
lcm.dat$region.cat[lcm.dat$region==4 | lcm.dat$region==5] = 2
lcm.dat$region.cat[lcm.dat$region>=6 & lcm.dat$region<=8] = 3
lcm.dat$region.cat[lcm.dat$region>8] = 4

lcm.dat$herd_size.cat = ifelse(lcm.dat$herd_size<=120,1,2)
lcm.dat$ams.cat = ifelse(lcm.dat$ams==0,1,2)
lcm.dat$prod.cat = lcm.dat$productiontype

results.herd = results.ams = results.prod = list()

#make the model

model.strep <- function(){

  # Priors for Se and Sp and the prevalence (p)
  for(i in 1:4){
    # test 1 Culture small herds: test 2 PCR small herds:
    # test 3 Culture large herds: test 4 PCR large herds;
    se[i] ~ dbeta(1,1)
    sp[i] ~ dbeta(1,1)
  }
  # The model
  # the first 4 populations are region 1+2+3+4 for small herds
  for(i in 1:4){
    p[i] ~ dbeta(1,1)
    pop[i,1:4] ~ dmulti(par[i,1:4],n[i])
    par[i,1] <- se[1]*se[2]*p[i] + (1-sp[1])*(1-sp[2])*(1-p[i])
    par[i,2] <- se[1]*(1-se[2])*p[i] + (1-sp[1])*(sp[2])*(1-p[i])
    par[i,3] <- (1-se[1])*(se[2])*p[i] + (sp[1])*(1-sp[2])*(1-p[i])
    par[i,4] <- (1-se[1])*(1-se[2])*p[i] + (sp[1])*(sp[2])*(1-p[i])
    n[i] <- sum(pop[i,1:4])
```

```
  }
  # the next 4 populations are region 1+2+3+4 for large herds
  for(i in 5:8){
    p[i] ~dbeta(1,1)
    pop[i,1:4] ~ dmulti(par[i,1:4],n[i])
    par[i,1] <- se[3]*se[4]*p[i] + (1-sp[3])*(1-sp[4])*(1-p[i])
    par[i,2] <- se[3]*(1-se[4])*p[i] + (1-sp[3])*(sp[4])*(1-p[i])
    par[i,3] <- (1-se[3])*(se[4])*p[i] + (sp[3])*(1-sp[4])*(1-p[i])
    par[i,4] <- (1-se[3])*(1-se[4])*p[i] + (sp[3])*(sp[4])*(1-p[i])
    n[i] <- sum(pop[i,1:4])
  }
  #Test whether Se cul in small herds differs from Se cul in large herds
  p.se[1] <- step(se[1] - se[3])

  #Test whether Se pcr in small herds differs from Se pcr in large herds
  p.se[2] <- step(se[2] - se[4])

  # Test whether Sp cul in small herds differs from Sp cul in large herds
  p.sp[1] <- step(sp[1] - sp[3])

  # Test whether Sp pcr in small herds differs from Sp pcr in large herds
  p.sp[2] <- step(sp[2] - sp[4])
}

#write model to a file
writeModel(model.strep,'strep.model.txt')

#Data in R
#the model will run specifically for each covariate for the 5 cut-offs

covs = c("herd.size","ams","prod")

for(cov in 1:length(covs)){

  cut.off = c(39.99,36.99,34.99,32.99,30.99)

  for(i in 1:length(cut.off)){

    print(c(covs[cov],cut.off[i]))

    lcm.dat$pcr_2 = ifelse(lcm.dat$ct_pcr<=cut.off[i],1,0)

    dat.mat = matrix(NA,ncol=4,nrow=8)

    for(h in 1:2){
```

```
      for(r in 1:4){

        all.pos = nrow(lcm.dat[lcm.dat$region.cat==r & lcm.dat[,21+cov]==h &
lcm.dat$gbs_cult==1 & lcm.dat$pcr_2==1,])
        pos.neg = nrow(lcm.dat[lcm.dat$region.cat==r & lcm.dat[,21+cov]==h &
lcm.dat$gbs_cult==1 & lcm.dat$pcr_2==0,])
        neg.pos = nrow(lcm.dat[lcm.dat$region.cat==r & lcm.dat[,21+cov]==h &
lcm.dat$gbs_cult==0 & lcm.dat$pcr_2==1,])
        all.neg = nrow(lcm.dat[lcm.dat$region.cat==r & lcm.dat[,21+cov]==h &
lcm.dat$gbs_cult==0 & lcm.dat$pcr_2==0,])

        if(h==1){dat.mat[r,] = c(all.pos,pos.neg,neg.pos,all.neg)}
        else{dat.mat[4+r,] = c(all.pos,pos.neg,neg.pos,all.neg)}

      }

    }

    sag.data = list(); sag.data[[1]] = dat.mat; names(sag.data) = "pop"

    #make the data for OpenBUGS
    bugsData(sag.data,fileName='StrepData.txt')

    # make 2 initial values chains

bugsInits(inits=list(list(se=rep(0.60,times=4),sp=rep(0.80,times=4),p=rep(0.10,t
imes=8))),numChains=1,'StrepInit1.txt')

bugsInits(inits=list(list(se=rep(0.70,times=4),sp=rep(0.90,times=4),p=rep(0.15,t
imes=8))),numChains=1,'StrepInit2.txt')

    # now check, load data, compile etc.
    modelCheck("strep.model.txt")          # check model file
    modelData("StrepData.txt")            # read data file
    modelCompile(numChains=2)            # compile model with 2 chains
    modelInits('StrepInit1.txt',1)  # read init data file
    modelInits('StrepInit2.txt',2)  # read init data file
    #modelGenInits()                # generate the missing initial values

    modelUpdate(10000)  #burn in

    samplesSet(c('se','sp','p','p.se','p.sp'))  #parameters to monitor

    modelUpdate(10000)  #10000 more iterations

    # check convergence - should check all, but we cheat
```

```
    samplesHistory('se',mfrow=c(1,1)) # plot the chain
    samplesHistory('sp',mfrow=c(1,1)) # plot the chain
    samplesHistory('p',mfrow=c(1,1))

    samplesBgr("se",mfrow=c(1,1))   #plot the bgr statistics - ratio should be
close to 1
    samplesBgr("sp",mfrow=c(1,1))
    samplesBgr("p",mfrow=c(1,1))


    samplesDensity("se",mfrow=c(1,1)) #density plots
    samplesDensity("sp",mfrow=c(1,1))
    samplesDensity("p",mfrow=c(1,1))

    if(cov==1){results.herd[[i]] = samplesStats("*")}   #the summarized results
    if(cov==2){results.ams[[i]] = samplesStats("*")}
    if(cov==3){results.prod[[i]] = samplesStats("*")}

  }

}

#Crude LCM model (without stratification)

results.herd = list()

#make the model

model.strep <- function(){

#Priors for Se and Sp and the prevalence (p)
  for(i in 1:2){

    #test 1 is Culture; test 2 is PCR

    se[i] ~ dbeta(1,1)
    sp[i] ~ dbeta(1,1)
  }
  # The model
  # the 4 populations of herds
  for(i in 1:4){
    p[i] ~ dbeta(1,1)
    pop[i,1:4] ~ dmulti(par[i,1:4],n[i])
    par[i,1] <- se[1]*se[2]*p[i] + (1-sp[1])*(1-sp[2])*(1-p[i])
    par[i,2] <- se[1]*(1-se[2])*p[i] + (1-sp[1])*(sp[2])*(1-p[i])
    par[i,3] <- (1-se[1])*(se[2])*p[i] + (sp[1])*(1-sp[2])*(1-p[i])
```

```
    par[i,4] <- (1-se[1])*(1-se[2])*p[i] + (sp[1])*(sp[2])*(1-p[i])
    n[i] <- sum(pop[i,1:4])
  }

  #Test whether Se cul differs with Se PCR
  p.se <- step(se[1] - se[2])

  # Test whether Sp cul differs with Sp PCR
  p.sp <- step(sp[1] - sp[2])


}

# write model to a file
writeModel(model.strep,'strep.model.txt')

#Data in R

cut.off = c(39.99,36.99,34.99,32.99,30.99)

for(i in 1:length(cut.off)){

  print(cut.off[i])

  lcm.dat$pcr_2 = ifelse(lcm.dat$ct_pcr<=cut.off[i],1,0)

  dat.mat = matrix(NA,ncol=4,nrow=4)

  for(r in 1:4){

    all.pos = nrow(lcm.dat[lcm.dat$region.cat==r & lcm.dat$gbs_cult==1 &
lcm.dat$pcr_2==1,])
    pos.neg = nrow(lcm.dat[lcm.dat$region.cat==r & lcm.dat$gbs_cult==1 &
lcm.dat$pcr_2==0,])
    neg.pos = nrow(lcm.dat[lcm.dat$region.cat==r & lcm.dat$gbs_cult==0 &
lcm.dat$pcr_2==1,])
    all.neg = nrow(lcm.dat[lcm.dat$region.cat==r  & lcm.dat$gbs_cult==0 &
lcm.dat$pcr_2==0,])

    dat.mat[r,] = c(all.pos,pos.neg,neg.pos,all.neg)

  }

  sag.data = list(); sag.data[[1]] = dat.mat; names(sag.data) = "pop"

  #make the data for OpenBUGS
```

```
  bugsData(sag.data,fileName='StrepData.txt')

  # make 2 initial values chains

bugsInits(inits=list(list(se=rep(0.60,times=2),sp=rep(0.80,times=2),p=rep(0.10,t
imes=4))),numChains=1,'StrepInit1.txt')

bugsInits(inits=list(list(se=rep(0.70,times=2),sp=rep(0.90,times=2),p=rep(0.15,t
imes=4))),numChains=1,'StrepInit2.txt')

  # now check, load data, compile etc.
  modelCheck("strep.model.txt")          # check model file
  modelData("StrepData.txt")            # read data file
  modelCompile(numChains=2)            # compile model with 2 chains
  modelInits('StrepInit1.txt',1)  # read init data file
  modelInits('StrepInit2.txt',2)  # read init data file
  #modelGenInits()                # generate the missing initial values

  modelUpdate(10000)  #burn in

  samplesSet(c('se','sp','p','p.se','p.sp'))  #parameters to monitor

  modelUpdate(10000)  #10000 more iterations

  results.herd[[i]] = samplesStats("*")   #the summarized results

}
```

CHAPTER 3a

```
rm(list=ls())

library(foreign)
library(MASS)

inc.dat = read.dta("F:/INCIDENCE/INCIDENCE.dta")[1:13]; names(inc.dat)

yrs = 1998:2009

#Compute Infection exit, recovery, non-infection exit and incidencE rates and
return 95% CI

#Counts
s.ent = i.ent = ie = rec = nie = inc = ini = ins = vector()

for(i in 1:10){
  s.ent[i] = nrow(subset(inc.dat,inc.dat[,i+3]==0 & is.na(inc.dat[,i+1]) &
is.na(inc.dat[,i+2])))
  i.ent[i] = nrow(subset(inc.dat,inc.dat[,i+3]==1 & is.na(inc.dat[,i+1]) &
is.na(inc.dat[,i+2])))
  rec[i] = nrow(subset(inc.dat,inc.dat[,i+2]==1 & inc.dat[,i+3]==0))
  inc[i] = nrow(subset(inc.dat,inc.dat[,i+2]==0 & inc.dat[,i+3]==1))

  if(i<10){
    ie[i] =  nrow(subset(inc.dat,inc.dat[,i+2]==1 & is.na(inc.dat[,i+3]) &
is.na(inc.dat[,i+4])))
    nie[i] = nrow(subset(inc.dat,inc.dat[,i+2]==0 & is.na(inc.dat[,i+3]) &
is.na(inc.dat[,i+4])))
  }
  else{
    ie[i] = nrow(subset(inc.dat,inc.dat[,i+2]==1 & is.na(inc.dat[,i+3])))
    nie[i] = nrow(subset(inc.dat,inc.dat[,i+2]==0 & is.na(inc.dat[,i+3])))
  }

  ini[i] = nrow(subset(inc.dat,inc.dat[,i+2]==1))
  ins[i] = nrow(subset(inc.dat,inc.dat[,i+2]==0))

}

#Compute a function that gives rates and returns 95% CI
rates = function(s.ent,i.ent,ie,rec,nie,inc,ini,ins){

  ani = ini + (0.5*((i.ent+inc)-(rec+ie))); ans = ins + (0.5*((s.ent+rec)-
(inc+nie)))
```

```
  rate.a = ie/ani; rate.b = rec/ani; rate.c = nie/ans; rate.d = inc/ans
  EF.a = exp(1.96/sqrt(ie)); EF.b = exp(1.96/sqrt(rec)); EF.c =
exp(1.96/sqrt(nie)); EF.d = exp(1.96/sqrt(inc))
  lcl.a = rate.a/EF.a; lcl.b=rate.b/EF.b; lcl.c=rate.c/EF.c; lcl.d=rate.d/EF.d
  ucl.a = rate.a*EF.a; ucl.b=rate.b*EF.b; ucl.c=rate.c*EF.c; ucl.d=rate.d*EF.d

  return(list(IEr=rate.a*100,RECr=rate.b*100,NIEr=rate.c*100,INCr=rate.d*100,

lcl.IE=lcl.a*100,lcl.REC=lcl.b*100,lcl.NIE=lcl.c*100,lcl.INC=lcl.d*100,

ucl.IE=ucl.a*100,ucl.REC=ucl.b*100,ucl.NIE=ucl.c*100,ucl.INC=ucl.d*100))

}

(RATES =
rates(s.ent=s.ent,i.ent=i.ent,ie=ie,rec=rec,nie=nie,inc=inc,ini=ini,ins=ins))

#Compute averages for entry,infection/non-infection and recovery rates plus
transmission parameter

pop.size = as.vector(apply(inc.dat[,3:12],2,function(x)sum(!is.na(x))))

ave.dat = data.frame(year=yrs[-
(1:2)],imi=inc,recoveries=rec,exsus=nie,exinf=ie,entsus=s.ent,

entinf=i.ent,susc=ins,infec=ini,pop.size=pop.size,time=rep(1:2,each=5))

ave.dat$ln_sin = log(ave.dat$susc*ave.dat$infec/ave.dat$pop.size)
ave.dat$ln_inf = log(ave.dat$infec); ave.dat$ln_susc = log(ave.dat$susc)
ave.dat$totent= apply(ave.dat[,6:7],1,sum); ave.dat$ln_N = log(ave.dat$pop.size)

beta.par = glm.nb(imi~offset(ln_sin),data=ave.dat) #beta parameter
#summary(beta.par)
exp(cbind(estimate=coef(beta.par),confint(beta.par)))

beta.test = glm.nb(imi~time + offset(ln_sin),data=ave.dat) #test whether beta
differs before and after 2005
summary(beta.test)

inf.exit.par = glm.nb(exinf~offset(ln_inf),data=ave.dat) # infectious exit rate
#summary(inf.exit.par)
exp(cbind(estimate=coef(inf.exit.par),confint(inf.exit.par)))

non.inf.exit.par = glm.nb(exsus~offset(ln_susc),data=ave.dat) # non-infectious
exit rate
#summary(non.inf.exit.par)
```

```
exp(cbind(estimate=coef(non.inf.exit.par),confint(non.inf.exit.par)))

rec.par = glm.nb(recoveries~offset(ln_inf),data=ave.dat) # recovery rate
#summary(rec.par)
exp(cbind(estimate=coef(rec.par),confint(rec.par)))

ent.par = glm.nb(totent~offset(ln_N),data=ave.dat) # entry rate
#summary(ent.par)
exp(cbind(estimate=coef(ent.par),confint(ent.par)))

(pop.sus = mean(ave.dat$entsus/ave.dat$totent)) #proportion of susceptible
entries

#Basic reproductive ratio
(R0.mean = as.vector(exp(coef(beta.par)))/(as.vector(exp(coef(rec.par))) +
as.vector(exp(coef(inf.exit.par)))))
(R0.lcl =
as.vector(exp(confint(beta.par)[1]))/(as.vector(exp(confint(rec.par)[1])) +
as.vector(exp(confint(inf.exit.par)[1]))))
(R0.ucl =
as.vector(exp(confint(beta.par)[2]))/(as.vector(exp(confint(rec.par)[2])) +
as.vector(exp(confint(inf.exit.par)[2]))))
```

CHAPTER 3b

```
rm(list=ls())

setwd("F:/INCIDENCE/Inc_data")

library(BRugs)

all.data = read.csv("all.dat.csv",header=T)

recip = function(prop,n.inf){
  pop = round((1/(prop/100))*n.inf,digits=0)
  return(pop.r=pop)
}

#MODEL

inc.prev <- function(){

  se[1] ~ dbeta(29.0522,14.201)
  sp[1] ~ dbeta(1258.868824,4.784987908)
  se[2] ~ dbeta(60.0066,3.9751)
  sp[2] ~ dbeta(306.1684,4.7065)

  for(i in 1:24){

    tp[i] ~ dbeta(1,1) #prev from 1966 to 1990: 1978 data are missing

    y[i] ~ dbin(ap[i],n[i])
    ap[i] <- se[1]*tp[i]+(1-sp[1])*(1-tp[i])

  }

  for(i in 25:41){

    #priors
    r[i-24] ~ dbeta(1,1)
    It[i-24] ~ dbeta(1,1) #1992 to 2008 - incidence - all culture
    tp[i] ~ dbeta(1,1)

    inc[i-24] ~ dbin(IO[i-24],inar[i-24])
    IO[i-24] <- (se[1]*sp[1]*(1-tp[i])*It[i-24] + (1-se[1])*tp[i]*(1-r[i-24]) +
(1-sp[1])*sp[1]*(1-tp[i])*(1-It[i-24]) + (1-se[1])*tp[i]*r[i-24])/
      (sp[1]*(1-tp[i]) + (1-se[1])*tp[i])

    y[i] ~ dbin(ap[i],n[i]) # will give prev from 1991 to 2007 - all culture
```

```
    ap[i] <- se[1]*tp[i]+(1-sp[1])*(1-tp[i])

  }

  for(i in 42:42){
    r[i-24] ~ dbeta(1,1)
    It[i-24] ~ dbeta(1,1) # incidence in 2009 by pcr
    tp[i] ~ dbeta(1,1) # prev in 2008 based on culture

    inc[i-24] ~ dbin(I0[i-24],inar[i-24])
    I0[i-24] <- (se[2]*sp[1]*(1-tp[i])*It[i-24] + (1-se[1])*tp[i]*(1-r[i-24]) +
(1-sp[2])*sp[1]*(1-tp[i])*(1-It[i-24]) + (1-se[1])*tp[i]*r[i-24])/
      (sp[1]*(1-tp[i]) + (1-se[1])*tp[i])

    y[i] ~ dbin(ap[i],n[i])
    ap[i] <- se[1]*tp[i]+(1-sp[1])*(1-tp[i])

  }

  for(i in 43:44){
    r[i-24] ~ dbeta(1,1)
    It[i-24] ~ dbeta(1,1) #incidence in 2010 and 2011 pcr
    tp[i] ~ dbeta(1,1) # prev in 2009 and 2010 pcr

    inc[i-24] ~ dbin(I0[i-24],inar[i-24])
    I0[i-24] <- (se[2]*sp[2]*(1-tp[i])*It[i-24] + (1-se[2])*tp[i]*(1-r[i-24]) +
(1-sp[2])*sp[2]*(1-tp[i])*(1-It[i-24]) + (1-se[2])*tp[i]*r[i-24])/
      (sp[2]*(1-tp[i]) + (1-se[2])*tp[i])

    y[i] ~ dbin(ap[i],n[i])
    ap[i] <- se[2]*tp[i]+(1-sp[2])*(1-tp[i])

  }

  for(i in 45:45){
    tp[i] ~ dbeta(1,1) #prev in 2011 pcr

    y[i] ~ dbin(ap[i],n[i])
    ap[i] <- se[2]*tp[i]+(1-sp[2])*(1-tp[i])

    }

}

#write model to a file
writeModel(inc.prev,'strep.model.txt')
```

```
#Data
cases = pop.r = vector() #Generate prevalence estimates

for(y in 1991:2011){ #1989-1990 data not complete

  if(y==1991){
    yr.all = all.data[all.data[,"year"]==y,]; yr.all =
yr.all[!duplicated(yr.all[,1]),][1:2]
    cases[(y-1991)+1] = sum(yr.all[,"test"],na.rm=T)
    pop.r[(y-1991)+1] = nrow(yr.all[complete.cases(yr.all),])
    colnames(yr.all)[2] = paste("test.",y,sep="")
  }
  else{
    yr.dat = all.data[all.data[,"year"]==y,]; yr.dat =
yr.dat[!duplicated(yr.dat[,1]),][1:2]
    cases[(y-1991)+1] = sum(yr.dat[,"test"],na.rm=T)
    pop.r[(y-1991)+1] = nrow(yr.dat[complete.cases(yr.dat),])

    colnames(yr.dat)[2] = paste("test.",y,sep="")
    yr.all = merge(yr.all,yr.dat,by="herd",all.x=T,all.y=T)
  }

}

#Prevalence data from 1966-1990
prop = c(3.2,3.1,2.9,2.7,2.5,2.6,2.5,2.5,2.6,2.5,2.3,2.1,2.3,1.9,1.6,
         2.8,2.7,2.0,1.5,1.5,1.2,1.0,0.9,1.1)
n.inf = c(1352,1363,1485,1476,1317,1365,1401,1269,1279,1205,1061,879,
          801,606,526,942,894,625,424,397,298,226,202,221)

pop = recip(prop=prop,n.inf=n.inf)

inc = pops = vector() #incidence estimates

for(n in 2:(ncol(yr.all)-1)){

  inc[n-1] = nrow(subset(yr.all,yr.all[,n]==0 & yr.all[,n+1]==1))
  pops[n-1] = nrow(subset(yr.all,yr.all[,n]==0 & !is.na(yr.all[,n+1])))

}

inc.prev.data = list(y=c(n.inf,cases),n=c(pop,pop.r),inc=inc,inar=pops)

bugsData(inc.prev.data,fileName='StrepData.txt')
```

```
bugsInits(inits=list(list(se=rep(0.60,times=2),sp=rep(0.80,times=2),tp=rep(0.1,t
imes=length(inc.prev.data$y)))),numChains=1,'StrepInit1.txt')
bugsInits(inits=list(list(se=rep(0.70,times=2),sp=rep(0.70,times=2),tp=rep(0.05,
times=length(inc.prev.data$y)))),numChains=1,'StrepInit2.txt')

#now check, load data, compile etc.
modelCheck("strep.model.txt") #check model file
modelData("StrepData.txt") # read data file
modelCompile(numChains=2) # compile model with 2 chains
modelInits('StrepInit1.txt',1) # read init data file
modelInits('StrepInit2.txt',2) # read init data file
modelGenInits() # generate the missing initial values

modelUpdate(20000)  #burn in

samplesSet(c('ap','tp','I0','It','se','sp'))

modelUpdate(100000) #100000 more iterations

# check convergence - should check all, but we cheat
#samplesHistory('se',mfrow=c(1,1)) # plot the chain,
#samplesHistory('sp',mfrow=c(1,1)) # plot the chain,
#samplesHistory('ap',mfrow=c(1,1))
#samplesHistory('tp',mfrow=c(1,1))
#samplesHistory('I0',mfrow=c(1,1))
#samplesHistory('It',mfrow=c(1,1))

#samplesBgr("se",mfrow=c(1,1))   #plot the bgr statistics - ratio should be
close to 1
#samplesBgr("sp",mfrow=c(1,1))
#samplesBgr("ap",mfrow=c(1,1))
#samplesBgr("tp",mfrow=c(1,1))
#samplesBgr("I0",mfrow=c(1,1))
#samplesBgr("It",mfrow=c(1,1))

#samplesDensity("se",mfrow=c(1,1)) #density plots
#samplesDensity("sp",mfrow=c(1,1))
#samplesDensity("ap",mfrow=c(1,1))
#samplesDensity("tp",mfrow=c(1,1))
#samplesDensity("I0",mfrow=c(1,1))
#samplesDensity("It",mfrow=c(1,1))

(results <- samplesStats("*")) #the summarized results

#Plot
```

```
x11(); Year = c(1966:1977,1979:2011); my.col = c("blue","red"); my.pch =
c(15,20)

minY = min(results$val2.5pc[90:134]*100, results$val2.5pc[21:40]*100)
maxY = max(results$val97.5pc[90:134]*100, results$val97.5pc[21:40]*100)

plot(Year,results$median[90:134]*100,pch=my.pch[1],col=my.col[1],xlim=c(1966,201
1),ylim=c(minY-0.5,maxY+0.5),cex.axis=0.8,cex.lab=1.2,
     xlab="Year",ylab="Frequency (%)",type='p',las=1,xaxt="n")
axis(1,Year,las=2)

for(i in 1:45){

arrows(Year[i],results$val2.5pc[90:134][i]*100,Year[i],results$val97.5pc[90:134]
[i]*100,lwd=1.5,code=3,angle=90,length=0.1,col=my.col[1])
}

points(Year[-(1:25)],results$median[21:40]*100,pch=my.pch[2],col=my.col[2])

for(i in 1:20){
  arrows(Year[-(1:25)][i],results$val2.5pc[21:40][i]*100,Year[-
(1:25)][i],results$val97.5pc[21:40][i]*100,lwd=1.5,code=3,angle=90,length=0.1,co
l=my.col[2])
}

legend("topleft",c("True prevalence","True incidence
risk"),pch=my.pch,col=my.col)

for(k in c(1,3,5)){abline(h=k,lty=4,col="darkgray")}

for(m in c(1966,1983,1991,2005,2009)){abline(v=m,lty=4,col="darkgray")}

text(x=1966,y=-0.5,labels=paste("Voluntary","\nsurveillance","\n&
control",sep=""),cex=0.65,pos=4)
text(x=1983,y=-0.5,labels=paste("Mandatory","\nsurveillance","\n&
control",sep=""),cex=0.65,pos=4)
text(x=1991,y=-0.5,labels=paste("Voluntary control","\n& movement
ban",sep=""),cex=0.65,pos=4)
text(x=2005,y=-0.5,labels=paste("Reversal of","\nmovement
ban",sep=""),cex=0.65,pos=4)
text(x=2009,y=-0.5,labels=paste("Screening","\nby PCR",sep=""),cex=0.65,pos=4)
```

CHAPTER 4

```
rm(list=ls())

setwd("C:/Dropbox/MARSHAL_FILES/MOVT_DATA")

library(igraph) #but could try igraph0
library(foreign)

#Create a function to compute assortativity
assortativity = function(graph){
  deg = degree(graph)
  deg.sq = deg^2
  m = ecount(graph)
  num1 = 0; num2 = 0; den = 0
  edges = get.edgelist(graph, names=FALSE)

  num1 = sum(deg[edges[,1]] * deg[edges[,2]])/m
  num2 = (sum(deg[edges[,1]] + deg[edges[,2]])/(2*m))^2
  den = sum(deg.sq[edges[,1]] + deg.sq[edges[,2]])/(2*m)

  return((num1-num2)/(den-num2))
}

#PREMISE NETWORK
Clustering_v = GSCsize_v = GWCsize_v = Av_path_v = Density_v = alpha.in_v =
alpha.out_v =
Assort.coef_v = Bet.herd.id = Clo.herd.id = Deg.herd.id = Bet.herd.type =
Clo.herd.type =
Deg.herd.type = Tot.dairy.counts = GSCsize_abs = GWCsize_abs = GSCsize_Prop.Mkt
=
GWCsize_Prop.Mkt = Nodes = Edges = vector()

Deg.dist.in_l = Deg.dist.out_l = list()

#Start and end dates of milk production data
dairy.data = read.dta("strep_prod.dta",warn.missing.labels=F)[c(1,4,5,6,9,10)]
colnames(dairy.data)[3] = "years"
dairy.data$start_date = as.Date(dairy.data$firstmilk,origin="1960-01-01")
dairy.data$end_date = as.Date(dairy.data$lastmilk,origin="1960-01-01")

#Beef data
beef.data = read.dta("beef_herds.dta")
beef.data[,3] = as.Date(beef.data[,3],origin="1960-01-01")
beef.data[,5] = as.Date(beef.data[,5],origin="1960-01-01")
```

```
for(year in 2000:2009){

  year.data = read.dta(paste(year,"_data.dta",sep=""))[c(8,9,14,15,19)]

  dat.sub = subset(dairy.data,years==year)

  for(months in 1:12){

    print(c(year,months))

    #start of the month
    my.date1 = as.Date(paste(months,"/1/",year,sep=""),format="%m/%d/%Y")

    #end of the month
    my.date2 = as.Date(paste(months,"/28/",year,sep=""),format="%m/%d/%Y")

    #Dairies between start and end of month
    dairy.ids = dat.sub[dat.sub[,7]<=my.date1 & dat.sub[,8]>=my.date2,1]
#dairies in prodn during month
    dairy.counts = length(dairy.ids)

    #Beef herds between start and end of month
    beef.counts = nrow(beef.data[beef.data[,3]<=my.date1 &
beef.data[,5]>=my.date2,])

    #subset movement data by month
    month.data = subset(year.data,month_id==months)

    #count no. of breeding, dealer, mkts, shows, pastures, hospitals in a month
    counts.breed =
length(unique(c(month.data[month.data[3]==41,1],month.data[month.data[4]==41,2])
))
    counts.dealer =
length(unique(c(month.data[month.data[3]==61,1],month.data[month.data[4]==61,2])
))
    counts.mkts =
length(unique(c(month.data[month.data[3]==62,1],month.data[month.data[4]==62,2])
))
    counts.shows =
length(unique(c(month.data[month.data[3]==65,1],month.data[month.data[4]==65,2])
))
    counts.pasture =
length(unique(c(month.data[month.data[3]==67,1],month.data[month.data[4]==67,2])
))
```

```
    counts.hos =
length(unique(c(month.data[month.data[3]==80,1],month.data[month.data[4]==80,2])
))
    counts.beef =
length(unique(c(month.data[month.data[3]==11,1],month.data[month.data[4]==11,2])
))
    counts.dairy =
length(unique(c(month.data[month.data[3]==14,1],month.data[month.data[4]==14,2])
))

    counts.other = counts.breed + counts.dealer + counts.mkts + counts.shows +
counts.pasture + counts.hos
    counts.total = counts.other + counts.beef + counts.dairy

    #Collapse data and show counts for each row pair
    collapsed.data =
aggregate(month.data[,1],list(month.data[,1],month.data[,2]),length)
    colnames(collapsed.data) = c('herd_id_from','herd_id_to','counts')

    # Adjacency Matrix
    Matrix = as.matrix(collapsed.data)

    graph.obj = graph.data.frame(Matrix[,1:2])

    #Number of monthly nodes and edges
    nodes = length(V(graph.obj))

    edges = length(E(graph.obj))

    #DESCRIPTIVE MEASURES IN A NETWORK
    #Components
    cl.strong = clusters(graph.obj, mode="strong")
    cl.weak = clusters(graph.obj, mode="weak")

    GSCsize = max(cl.strong$csize) #size of GSC
    GWCsize = max(cl.weak$csize)    #size of GWC

    GSCsize_prop = GSCsize/(counts.other+dairy.counts+beef.counts) #Proportion
of nodes in GSC
    GWCsize_prop = GWCsize/(counts.other+dairy.counts+beef.counts)   #Proportion
of nodes in GWC

    #Herd Ids belonging to Giant strong/weak components
    strong.loc = which(which.max(cl.strong$csize)==cl.strong$membership) #gives
u positions of the vertices in GSC
    vert.strong = V(graph.obj)[strong.loc] #vertices in giant strong component
```

```
    weak.loc = which(which.max(cl.weak$csize)==cl.weak$membership)
    vert.weak = V(graph.obj)[weak.loc]

    #Clustering coefficient
    cluster.coeff = transitivity(graph.obj,type="global") #Proportion of triads
in the network

    #path length
    Av_path = average.path.length(graph.obj)

    #Density
    Dens = graph.density(graph.obj,loops=F)

    #Assortativity - BASED on Pearson's correlation coefficient - Negative vals
show disassortative mixing - scale free behavior
    Assort.coef = assortativity(graph.obj)

    #Betweenness, closeness and total degree
    Betwn = igraph::betweenness(graph.obj)
    Close = igraph::closeness(graph.obj,mode="total")
    Deg.all = igraph::degree(graph.obj,mode="total",loops=F)

    #Checking whether in.degrees and out.degrees follow power law distribution
    Deg.in = igraph::degree(graph.obj,mode="in",loops=F)
    Deg.out = igraph::degree(graph.obj,mode="out",loops=F)

    #Plfit function gives alpha values using an xmin that minimises the
Kolmogorov-Smirnov goodness of fit statistic (D)
    alpha.in = stats4::coef(power.law.fit(Deg.in+1,implementation="R.mle"))
#power law exponent- +1 ensures that all 0's have 1's
    alpha.out = stats4::coef(power.law.fit(Deg.out+1,implementation="R.mle"))
#values of 2<alpha<3 accepted as fitting power laws

    #degree dist in all the years
    Deg.dist.in = degree.distribution(graph.obj,mode="in",cumulative=T,loops=F)
    Deg.dist.out =
degree.distribution(graph.obj,mode="out",cumulative=T,loops=F)

    #Calculate the median betwn, close and deg score for each premise type in
each month and then..
    #get the median of medians and range of medians of these scores
    herd.type = vector()

    for(h.ty in 1:length(V(graph.obj))){
      herd.type.from =
month.data[month.data[,1]==as.numeric(V(graph.obj)$name[h.ty]),][1,][,3]
```

```
      herd.type.to =
month.data[month.data[,2]==as.numeric(V(graph.obj)$name[h.ty]),][1,][,4]
      herd.type[h.ty] =
ifelse(is.na(herd.type.from),herd.type.to,herd.type.from)
    }

    prem.type = as.numeric(levels(as.factor(herd.type)))

    #Betweenness
    bet.score = clo.score = deg.score = vector()

    for(x in 1:length(prem.type)){
      bet.score[x] = median(Betwn[herd.type==prem.type[x]])
      clo.score[x] = median(Close[herd.type==prem.type[x]])
      deg.score[x] = median(Deg.all[herd.type==prem.type[x]])
    }

    prem.bet = cbind(prem.type,bet.score);colnames(prem.bet)[2] =
paste("mon",(year-2000)*12+months,sep="")
    prem.clo = cbind(prem.type,clo.score);colnames(prem.clo)[2] =
paste("mon",(year-2000)*12+months,sep="")
    prem.deg = cbind(prem.type,deg.score);colnames(prem.deg)[2] =
paste("mon",(year-2000)*12+months,sep="")

    #Assessing small world properties
    cluster.erdos = av.path.erdos = vector()

    for(ran in 1:1000){
      graph.erdos.obj =
erdos.renyi.game(n=nodes,p.or.m=edges,type="gnm",directed=T,loops=F) #random
graph with same nos of nodes and edges as observed network
      cluster.erdos[ran] = transitivity(graph.erdos.obj,type="global") #cluster
coef of random graph
      av.path.erdos[ran] = average.path.length(graph.erdos.obj) #ave. path
length of random graph
    }

    cluster.erdos = median(cluster.erdos)
    av.path.erdos = median(av.path.erdos)

    cl.coef.test = (cluster.coeff/cluster.erdos) >= 20
    av.path.test = Av_path <= av.path.erdos
    cl.av.test = cbind(cl.coef.test,av.path.test)

    #Remove all markets from month data
    minus.mkt.data = month.data[!month.data[,3]==62 | month.data[,4]==62,]
```

```
    collapsed.data.mkt =
aggregate(minus.mkt.data[,1],list(minus.mkt.data[,1],minus.mkt.data[,2]),length)
    colnames(collapsed.data.mkt) = c('herd_id_from','herd_id_to','counts')

    # Adjacency Matrix
    Matrix.mkt = as.matrix(collapsed.data.mkt)
    graph.obj.mkt = graph.data.frame(Matrix.mkt[,2:3])
    GSCsize.mkt = max(clusters(graph.obj.mkt, mode="strong")$csize) #size of GSC
    GWCsize.mkt = max(clusters(graph.obj.mkt, mode="weak")$csize) #size of GWC
    GSCsize_prop.mkt = GSCsize.mkt/(counts.other+dairy.counts+beef.counts)
    GWCsize_prop.mkt = GWCsize.mkt/(counts.other+dairy.counts+beef.counts)
#Proportion of nodes in GSC

    #Store the results
    Clustering_v[(year-2000)*12+months] = cluster.coeff
    GSCsize_v[(year-2000)*12+months] = GSCsize_prop
    GWCsize_v[(year-2000)*12+months] = GWCsize_prop
    Av_path_v[(year-2000)*12+months] = Av_path
    Density_v[(year-2000)*12+months] = Dens
    alpha.in_v[(year-2000)*12+months] = alpha.in
    alpha.out_v[(year-2000)*12+months] = alpha.out
    Assort.coef_v[(year-2000)*12+months] = Assort.coef
    Deg.dist.in_l[[(year-2000)*12+months]] = Deg.dist.in
    Deg.dist.out_l[[(year-2000)*12+months]] = Deg.dist.out
    Tot.dairy.counts[(year-2000)*12+months] = dairy.counts

    GSCsize_abs[(year-2000)*12+months] = GSCsize
    GWCsize_abs[(year-2000)*12+months] = GWCsize
    GSCsize_Prop.Mkt[(year-2000)*12+months] = GSCsize_prop.mkt
    GWCsize_Prop.Mkt[(year-2000)*12+months] = GWCsize_prop.mkt
    Nodes[(year-2000)*12+months] = nodes
    Edges[(year-2000)*12+months] = edges

    if(year==2000 & months==1){
      premise.betwn = prem.bet
      premise.clo = prem.clo
      premise.deg = prem.deg
      small.wd.test = cl.av.test
    }

    else{
      premise.betwn =
merge(premise.betwn,prem.bet,by="prem.type",all.x=T,all.y=T)
      premise.clo = merge(premise.clo,prem.clo,by="prem.type",all.x=T,all.y=T)
      premise.deg = merge(premise.deg,prem.deg,by="prem.type",all.x=T,all.y=T)
      small.wd.test = rbind(small.wd.test,cl.av.test)
```

```
    }

  }

}

premise.betwn[,1] =
c("beef","dairy","breeder","dealer","market","show","pasture","hospital")
premise.clo[,1] =
c("beef","dairy","breeder","dealer","market","show","pasture","hospital")
premise.deg[,1] =
c("beef","dairy","breeder","dealer","market","show","pasture","hospital")

(betweenness.score =
apply(premise.betwn[,2:ncol(premise.betwn)],1,median,na.rm=T)) #for the FUNCTION
part, Type MEDIAN and then RANGE
(closeness.score = apply(premise.clo[,2:ncol(premise.betwn)],1,median,na.rm=T))
(degree.score = apply(premise.deg[,2:ncol(premise.betwn)],1,median,na.rm=T))
```

CHAPTER 5

```
rm(list=ls())

library(foreign)
library(lme4)
library(sparr)
library(spatstat)
library(geoR)

#Computing risk periods
setwd("E:/DENMARK_ARTICLES/MASTITIS_ARTICLES/B_STREP_DATA/STATA")

all.data = read.dta("STREP_1989-2011.dta")

colnames(all.data)[1] = "herd"

herds = risk.herd = list()

for(y in 1999:2009){

  if(y==1999){
    dat.all = all.data[all.data[,5]==y,c(1,7)]; dat.all =
dat.all[!duplicated(dat.all[,1]),]; rownames(dat.all) = NULL
    dat.all[,2] = as.Date(dat.all[,2],origin="1960-01-01"); colnames(dat.all)[2]
= paste("test_",y,sep="")
    herds[[(y-1999)+1]] = dat.all
  }

  else{
    dat.yr = all.data[all.data[,5]==y,c(1,7)]; dat.yr =
dat.yr[!duplicated(dat.yr[,1]),]; rownames(dat.yr) = NULL
    dat.yr[,2] = as.Date(dat.yr[,2],origin="1960-01-01"); colnames(dat.yr)[2] =
paste("test_",y,sep="")
    herds[[(y-1999)+1]] = dat.yr

    dat = merge(herds[[y-1999]],herds[[(y-1999)+1]],by="herd",all=T)
    dat$mon = (dat[,3] - dat[,2])/30; class(dat$mon) = "numeric";
colnames(dat)[4] = "month"; dat = dat[,c(1,4)]


write.csv(dat,file=paste("C:/Dropbox/MARSHAL_FILES/MODEL/risk.per_",y,".csv",sep
=""),row.names=F)

    if(y==2000){box.data.all = data.frame(year=y,months=dat[,2])}
    if(y!=2000){box.data = data.frame(year=y,months=dat[,2])
```

```
                box.data.all = rbind(box.data.all,box.data)}

  }

}

setwd("C:/Dropbox/MARSHAL_FILES/MODEL")

inc.data = read.dta("incidence.dta")[c(1,3:13)]
GIS.data = read.dta("GIS.dta")[1:3]

regions = read.csv("Geoinfo.csv",header=T)[c(1,9)]; colnames(regions) =
c("herd","region")
regions$region.cat[regions$region<=3] = 1
regions$region.cat[regions$region==4 | regions$region==5] = 2
regions$region.cat[regions$region>=6 & regions$region<=8] = 3
regions$region.cat[regions$region>=9] = 4
regions = regions[-2]

#Vectors
yr = 1999:2008
log.movt = log.base = gof.values = lwr.movt = upr.movt = lwr.base = upr.base =
check.NAs = c()

#Run year-specific mixed effects logistic regression models to compute the
annual baseline and movt risks
for(col in 1:10){

  print(yr[col]+1)

  new.data = inc.data[,c(1,(col+1):(col+2))];new.data =
new.data[complete.cases(new.data),];new.data = new.data[new.data[,2]==0,][-2]

  prev.mov.data =
read.dta(paste(yr[col],"_data.dta",sep=""),warn.missing.labels=F)

  agg.data =
aggregate(prev.mov.data$herd_id_from,list(prev.mov.data$herd_id_from,prev.mov.da
ta$herd_id_to),length)

  agg.data = agg.data[!agg.data[,1]==agg.data[,2],] #remove rows that animals
moved within the same premise

  colnames(agg.data) = c("herd_id_from","herd_id_to","counts");
rownames(agg.data) = NULL
```

```
  count.data = data.frame(herd = unique(agg.data[,2]),counts =
as.vector(tapply(agg.data$counts,agg.data$herd_id_to,sum)))

  param.data = merge(new.data,count.data,by="herd",all.x=T);
param.data[is.na(param.data[,3]),3] = 0 #if counts is NA then replace with 0

  param.data$movt = ifelse(param.data[,3]>0,1,0)

  #Add risk period to data in the "Bugs_File.R"
  risk.period = read.csv(paste("risk.per_",yr[col]+1,".csv",sep=""),header=T)
  param.data = merge(param.data,risk.period,by="herd",all.x=T)

  #Add x and y coordinates for spatial analysis
  colnames(GIS.data)[1] = "herd"
  param.data = merge(param.data,GIS.data,by="herd",all.x=T)

  param.data = param.data[!param.data[,1]==57373,]; check.NAs[col] =
any(!complete.cases(param.data[,c(6,7)]))

  #Add month as centered variable
  param.data$month.cent = param.data$month - 12

  #Add region as variable - random effect in the model
  param.data = merge(param.data,regions,by="herd",all.x=T)

  #Logistic model
  outcome = assign(paste("cul_",yr[col]+1,sep=""),param.data[,2]) #remove
characters from object eg "cul_2000" to cul_2000

  par.glm.full = glmer(outcome~movt + month.cent +
(1|region.cat),family="binomial",data=param.data)

  log.movt[col] = coef(summary(par.glm.full))[2,1]
  log.base[col] = coef(summary(par.glm.full))[1,1]
  lwr.movt[col] = coef(summary(par.glm.full))[2,1] +
qnorm(.025)*coef(summary(par.glm.full))[2,2]
  upr.movt[col] = coef(summary(par.glm.full))[2,1] +
qnorm(.975)*coef(summary(par.glm.full))[2,2]
  lwr.base[col] = coef(summary(par.glm.full))[1,1] +
qnorm(.025)*coef(summary(par.glm.full))[1,2]
  upr.base[col] = coef(summary(par.glm.full))[1,1] +
qnorm(.975)*coef(summary(par.glm.full))[1,2]

  #Residuals
  param.data$res = residuals(par.glm.full) #pearson standardised residuals
  param.data$probs = fitted(par.glm.full)
```

```
  write.csv(param.data,file=paste("param_",yr[col]+1,".csv",sep=""),row.names=F)

}

#Create a function to compute the baseline and movt risks and their 95%
confidence bounds
risk.model = function(a,b,c,d,e,f){

  logodds = a+b
  risk.exp = exp(logodds)/(1+exp(logodds))
  risk.unexp = exp(a)/(1+exp(a))
  base.lwr = exp(c)/(1+exp(c))
  base.upr = exp(d)/(1+exp(d))
  att.risk = risk.exp - risk.unexp #mean risk for movt
  movt.lwr = exp(c+e)/(1+exp(c+e)) - base.lwr
  movt.upr = exp(d+f)/(1+exp(d+f)) - base.upr

  return(list(base.rsk=risk.unexp,lwr.base.rsk=base.lwr,upr.base.rsk=base.upr,
              movt.risk=att.risk,lwr.movt.rsk=movt.lwr,upr.movt.rsk=movt.upr))
}

(risks =
risk.model(a=log.base,b=log.movt,c=lwr.base,d=upr.base,e=lwr.movt,f=upr.movt))
#risk per year


#Plot of Background risk and movt-related risk
my.col = c("red","green")
my.pch = c(15,17)
Year = 2000:2009; yr.movt =
c(1999.942,2000.891,2002.201,2003.064,2004.090,2005.132,2005.970,2007.033,2008.1
92,2009.152) #jitter(Year,amount=0.224)

tiff(file="F:/Spatial_work/SUBMIT/Fig_4.tiff",units='cm',width=17.8,height=12,re
s=300,pointsize=7) # Specify width as given in the paper.
#Pointsize can be useful to play around with.

plot(Year,risks$base.rsk*100,pch=my.pch[1],col=my.col[1],ylim=c(min(risks$lwr.mo
vt.rsk)*100-0.5,max(risks$upr.base.rsk)*100+0.5),cex.axis=0.8,cex.lab=1.2,
     xlab="Year",ylab="Incidence per 100 herds per year",type='p',las=1)
for(i in 1:10){

arrows(Year[i],risks$lwr.base.rsk[i]*100,Year[i],risks$upr.base.rsk[i]*100,lwd=1
.5,code=3,angle=90,length=0.1,col=my.col[1])
}
```

```
par(new=T)

plot(yr.movt,risks$movt.risk*100,pch=my.pch[2],col=my.col[2],ylim=c(min(risks$lw
r.movt.rsk)*100-0.5,max(risks$upr.base.rsk)*100+0.5),cex.axis=0.8,cex.lab=1.2,
     xlab="Year",ylab="Incidence per 100 herds per
year",type='p',las=1,xaxt="n")
for(i in 1:10){

arrows(yr.movt[i],risks$lwr.movt.rsk[i]*100,yr.movt[i],risks$upr.movt.rsk[i]*100
,lwd=2,code=3,lty="dotted",angle=90,length=0.1,col=my.col[2])
}

legend("topleft",c("Baseline risk","movement-related
risk"),lwd=c(1.5,2),lty=c("solid","dotted"),pch=my.pch,col=my.col)

dev.off()

#Checking whether risk differs between 2000-2004 and 2005-2009
#First merge the data
for(year in 2000:2009){

  if(year==2000){
    yr.dat.all = read.csv(paste("param_",year,".csv",sep=""),header=T);
colnames(yr.dat.all)[2] = "culture"
    yr.dat.all$yrs = year
    yr.dat.all$per = 1
  }
  else{yr.dat = read.csv(paste("param_",year,".csv",sep=""),header=T);
colnames(yr.dat)[2] = "culture"
       yr.dat$yrs = year
       if(year<=2004){yr.dat$per = 1}
       if(year>2004){yr.dat$per = 2}
       yr.dat.all = rbind(yr.dat.all,yr.dat)
  }

}

#Sort data by herd
yr.dat.all = yr.dat.all[order(yr.dat.all[,1]),]

#run a repeated measures analysis with region and herd as random effect to
assess period differences
#must first convert region.cat and herd as factors otherwise R will output error
- not necessary with single random effect - "herd"
yr.dat.all[c("herd","region.cat")] =
lapply(yr.dat.all[c("herd","region.cat")],as.factor)
```

```
ran.model = glmer(culture~movt + month.cent + as.factor(per) +
(1|region.cat/herd),family="binomial",data=yr.dat.all)

summary(ran.model)

#Descriptive stats
for(yr in 2000:2009){

  pars.data = read.csv(paste("param_",yr,".csv",sep=""),header=T)

  dat = data.frame(year = yr,new.c = sum(pars.data[,2]),pop.ris =
nrow(pars.data),inc = mean(pars.data[,2])*100, movt = mean(pars.data[,4])*100)

  if(yr==2000){dat.all = dat}
  if(yr!=2000){dat.all = rbind(dat.all,dat)}

  if(yr==2009){
    rs.periods =
data.frame(med.per=as.vector(tapply(box.data.all$months,box.data.all$year,median
,na.rm=T)),

iqr=as.vector(tapply(box.data.all$months,box.data.all$year,IQR,na.rm=T)))

    des.dat = cbind(dat.all,rs.periods,as.data.frame(risks)*100);
print(des.dat)}

}

#Estimation of the bandwidth for kernel smoothing - "h"
all.x=NULL; all.y=NULL

for(i in 2000:2009){
  arcgis = read.csv(paste("param_",i,".csv",sep=""),header=T)[11:12]
  range.x = range(arcgis[,1]); range.y = range(arcgis[,2])
  all.x = c(all.x,range.x); all.y = c(all.y,range.y)
}

h = c()

for(y in 2000:2009){

  print(y)

  arcgis = read.csv(paste("param_",y,".csv",sep=""),header=T)
```

```
  cases = arcgis[arcgis[,2]==1,(11:12)]; cases =
as.ppp(cases,c(range(all.x),range(all.y)))
  controls = arcgis[arcgis[,2]==0,(11:12)]; controls =
as.ppp(controls,c(range(all.x),range(all.y)))

  h[(y-2000)+1] =
LSCV.risk(cases=cases,controls=controls,hlim=c(0,50000),res=1000,method="kelsall
-diggle")

}

median(h)

#Plot a semivariogram of model residuals
par(mfrow=c(2,5)); par(mar=c(3,3,0.7,0.5),mgp=c(2,0.7,0))

for(y in 2000:2009){ #omnidirectional (all directions)

  arcgis =
read.csv(paste("DATA/param_",y,".csv",sep=""),header=T)[c("xcor_to","ycor_to","r
es")]

  #Change distances to kms
  arcgis[,1] = arcgis[,1]/1000; arcgis[,2] = arcgis[,2]/1000

  geo.dat = as.geodata(arcgis,coords.col=1:2,data.col=3)
  dat.v <- variog(geo.dat,max.dist=50)

  dat.env <-variog.mc.env(geo.dat,obj=dat.v,nsim=499)

  plot(dat.v,env=dat.env,xlab="distance (km)",col="red")

  legend("bottomleft",paste("Yr:",y,sep=" "))

}

#Inhomogeneous K function
#K difference envelope function
diff.envelope <- function(envelopeA, envelopeB) {
  nsim = ncol(envelopeA) - 1
  sim.diffs = NULL
  simsA = as.data.frame(envelopeA)
  simsB = as.data.frame(envelopeB)
  for (sim in 1:nsim) {
    sim.diff = simsA[,(sim+1)] - simsB[,(sim+1)]
    sim.diffs = cbind(sim.diffs, sim.diff)
```

```
  }
  lower = apply(sim.diffs, 1, min)
  upper = apply(sim.diffs, 1, max)
  return(data.frame(r = simsA$r, lower = lower, upper = upper))
}

par(mfrow=c(2,5)); par(mar=c(3,3,0.7,0.5),mgp=c(2,0.7,0))

for(y in 2000:2009){

  arcgis = read.csv(paste("DATA/param_",y,".csv",sep=""),header=T)

  cases = arcgis[arcgis[,2]==1,(11:12)]; case.pp =
ppp(cases[,1]/1000,cases[,2]/1000,range(arcgis[,11]/1000),range(arcgis[,12]/1000
),unitname=c("kilometre","kilometres"))
  pop.pp =
ppp(arcgis[,11]/1000,arcgis[,12]/1000,range(arcgis[,11]/1000),range(arcgis[,12]/
1000),unitname=c("kilometre","kilometres"))

  (max(arcgis[,12])/1000 - min(arcgis[,12])/1000)*0.5 #s should be NOT GREATER
THAN (so can be less than) half length of shorter side of rectangular area

  case.khat = Kinhom(case.pp,r=seq(0,50,length=50),correction="border") #can
change to "translate" with nlarge=Inf
  pop.khat = Kinhom(pop.pp,r=seq(0,50,length=50),correction="border")

  diff.k = case.khat$border - pop.khat$border

  case.envelope = envelope(case.pp,Kinhom,nsim=499,correction="border")
  pop.envelope = envelope(pop.pp,Kinhom,nsim=499,correction="border")

  case.pop.env = diff.envelope(case.envelope,pop.envelope)

  plot(case.khat$r, diff.k, type='o', ylim=c(-
(max(diff.k)+3000),max(diff.k)+3000),ylab='K(case) - K(pop)',xlab='Distance
(km)',col="red")
  lines(case.pop.env$r, case.pop.env$upper, lty=2)
  lines(case.pop.env$r, case.pop.env$lower, lty=2)
  abline(0,0,lty=4)

  legend("bottomleft",paste("Yr:",y,sep=" "))

}
```